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Dissertação

Pharmacogenomics of Drug Addiction

Dissertação apresentada à Faculdade de Farmácia da Universidade de Coimbra, para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Farmacêutica, realizada sob a orientação científica da Professora Doutora Maria Manuela Monteiro Grazina (Faculdade de Medicina da Universidade de Coimbra) e orientação interna do Professor Doutor Sérgio Simões (Faculdade de Farmácia da Universidade de Coimbra).

Carolina Macedo, 2014

For every complex problem there is an answer that is clear, simple, and wrong.

H. L. Mecken

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Pharmacogenomics of Addiction

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Abstract

Drug addiction is a chronic disease which affects millions of people worldwide with critical social and economical impact, besides the health burden. Repetitive exposure to drugs of abuse induces long-lasting neuroadaptative changes that promote drug-seeking behaviors.

The causes of vulnerability to addiction, although its complexity, have been pointed to be in association with environmental, social and/or genetic factors.

Pharmacogenetics and more recently pharmacogenomics developments with technical genetic resources, such as candidate gene and genome-wide analysis approaches, have played an important role unraveling the possible responsible genetic variants, like SNP or VNTR that may influence the vulnerability or having a protective effect in chemical dependence.

Since drug addiction is a complex disease spectrum, genetic results may be seen as contradictory in some studies, but some genetic variants have been proven to be consistently associated to disease. For example, 136A allele of ADH4 gene, coding for alcohol dehydrogenase, has been associated with increased susceptibility to alcohol dependency while the ADH1B*2 variant has shown to confer a protective effect for alcohol dependence.

The use of Next Generation Sequencing (NGS) platforms allowing massively parallel sequencing for assessing entire genome in a few days will probably grow, with a widespread use for obtaining a huge amount of genetic information as a powerful tool for deeper understanding and for development of novel therapeutic approaches to drug addiction.

Keywords: drug addiction, genetic variations, pharmacogenetics, pharmacogenomics.

Resumo

A dependência de drogas é uma doença crónica que afecta milhões de pessoas em todo o mundo com impacto social e económico crítico, além do problema de saúde em si. A exposição repetida ao abuso de drogas induz alterações neuroadaptativas duradouras que promovem comportamentos de busca de drogas.

As causas da vulnerabilidade para a dependência, apesar da sua complexidade, têm sido apontados para a associação com factores ambientais, sociais e / ou genéticas.

A farmacogenética e, mais recentemente desenvolvimentos na farmacogenómica com recursos técnicos genéticos, como as abordagens de gene candidato e genome-wide analysis, têm desempenhado um papel importante para desvendar as possíveis variantes genéticas responsáveis, como SNP ou VNTR que podem influenciar a vulnerabilidade ou ter um efeito protector na dependência química.

Como a dependência de drogas é uma doença complexa, os resultados genéticos podem ser considerados contraditória em alguns estudos, mas algumas variantes genéticas têm sido consistentemente associadas à doença. Por exemplo, o alelo 136A do gene ADH4, que codifica para a álcool-desidrogenase, tem sido associado com um aumento da susceptibilidade à dependência do álcool, enquanto a variante ADH1B * 2 demonstrou conferir um efeito protector para a dependência do álcool.

O uso de sequenciamento de plataformas de próxima geração (NGS) permitindo sequenciamento paralelo em massa para avaliar o genoma inteiro em poucos dias, irá provavelmente crescer, com um uso generalizado para a obtenção de uma enorme quantidade de informação genética como uma ferramenta poderosa para a compreensão mais profunda e para o desenvolvimento de novas abordagens terapêuticas para dependência de drogas.

Palavras-chave: dependência, drogas, variações genéticas, farmacogenética, farmacogenómica.

Abbreviations

DA	Dopamine
GABA	γ-aminobutyric acid
MFB	Medial Forebrain Bundle
MPC	Medial Prefrontal Cortex
NAc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
NGS	Next Generation Sequencing
PFC	Prefrontal Cortex
SN	*Substantia nigra*
VP	*Ventral Pallidum*
VTA	Ventral Tegmental Area

1. Introduction

Drug addiction, also known as substance dependence, is a chronically relapsing disorder characterized by: (i) compulsion to seek and take a drug despite significant harmful consequences, (ii) loss of control in limiting intake and recurrent failure to control the behavior and (iii) emergence of a negative emotional state (e.g. dysphoria, anxiety, irritability) when access to the drug is prevented. According to DSM-5, craving has been added as a new criterion for the diagnosis of substance abuse. [1-4]

2. Neurobiology of addiction

In individuals who are vulnerable to addiction, repetitive exposure to the agent induces long-lasting neuroadaptative changes that further promote drug-seeking behaviors and ultimately lead to persistent and uncontrolled patterns of use. These neuroadaptative changes are the bases for the tolerance, craving and withdrawal and lead to a motivational shift. [5]

Family history studies indicate that biological relatives of an individual who has been diagnosed with psychoactive substance dependence, bulimia, pathological gambling, or sexual addiction are at significantly higher risk, compared to the general population, to develop, at some point in their lives, one of these disorders. [2]

The reward pathway of the mammalian brain consists of synaptically interconnected neurons, which link the ventral tegmental area (VTA), nucleus accumbens (NAc), ventral pallidum (VP), and medial prefrontal cortex (MPFC). This circuit is strongly implicated in the neural processes underlying drug addiction, and its inhibition is implicated in such phenomena as withdrawal dysphoria and dysphoria-mediated drug craving. [6]

Exposure and access to hedonic stimuli, such like addictive drugs, results in the pleasurable, positively reinforcing effects of the drug and also ‘desire’ for it when drug is not present. One of the most important brain areas for reinforcement and pleasure is the NAc in the forebrain region. It receives input from dopamine-producing cells in the midbrain called the ventral tegmental area (VTA). In fact, the VTA–NAc pathway seems to be a site where virtually all drugs of abuse converge to produce their acute reward signals. The VTA contains dopaminergic cells that project to the frontal cortex and limbic system. Release of dopamine into the frontal cortex and NAc results in the subjective experience of pleasure. [7 – 9]

It is known that the ‘first-stage’ neurons originate from an unrelated group of ventral limbic forebrain loci termed the “anterior bed nuclei” of the medial forebrain bundle (MFB). These

'first-stage' neurons are myelinated and moderately fast-conducting, and they project posteriorly through the MFB to synapse on VTA dopaminergic cells. The 'second-stage' dopamine (DA) releasing neurons project anteriorly within the MFB to synapse in the NAc. From NAc, 'third-stage' enkephalinergic neurons carry the reward signal to VP. This 'third-stage' pathway appears to be critical for the phenotype expression of reward-related and incentive- related behaviors. A portion of the 'third-stage' pathway consists of enkephalinergic NAc projection neurons which co-localize with γ-aminobutyric acid (GABA) as a co-transmitter. [6]

The GABAergic and glutamatergic neural inputs into this core reward system have been recognized as critically important in the regulation of reward processes and reward-driven behaviors. [6]

The acute rewarding properties of psychostimulant drugs have long been known to depend on activation of the mesolimbic DA system and dopaminergic neuronal projections have been identified as the central component of this brain reward system. They extend from the VTA of the midbrain to parts of the limbic system, especially to the NAc shell and the frontal cortex. Both natural stimuli and several, but not all, substances of abuse - most prominently cocaine, amphetamine, and opiates, are able to increase the release of DA in the NAc. The firing of dopaminergic neurons in the VTA is usually under the control of GABAergic γ - amino-butyric acid interneurons. Dopamine is released when the reward is achieved in addition to the presence stimuli that predict recompense. [6, 7, 11]

Addictive drugs activate the above-mentioned brain reward processes. Such drugs appear to activate the 'second-stage' DA neurons of the VTA/NAc axis, thus, producing the pleasurable/ euphoric effects. If a drug activates the VTA system and increases dopamine in the NAc, it will cause reinforcement and addiction. However, the mechanisms of this effect and the magnitude of increased dopamine levels in these areas are often different. For example, some drugs, such as the amphetamines, increase release of dopamine from presynaptic terminals in the NAc. Certain drugs, such as cocaine, block the reuptake of synaptic dopamine into the presynaptic neurons. Other drugs of abuse, such as alcohol, act on the cell bodies in the ventral tegmentum that produce DA. Addictive opiates, such as heroin and oxycodone, inhibit GABA cells that surround and normally suppress VTA cell dopaminergic activity. Not all drugs activate the dopaminergic system to the same extent and, therefore, they have different addictive potentials. [6, 8]

Different types of drugs will elicit distinct responses. Cocaine and amphetamines activate the release of dopamine in the NAc and amygdala via direct actions on dopamine terminals.

Opioids activate opioid receptors in the VTA, NAc, and amygdala via direct actions on interneurons. Opioids facilitate the release of DA in NAc via an action either in the ventral tegmental area or the nucleus accumbens. But also it has been hypothesized that it may activate elements independent of the dopamine system. Alcohol activates GABAA receptors in the VTA, NAc, and amygdala via either direct action at the GABAA receptor or through indirect release of GABA. Alcohol facilitates the release of dopamine in the nucleus accumbens either in the ventral tegmental area or the nucleus accumbens. Nicotine activates nicotinic acetylcholine receptors in the ventral tegmental area, nucleus accumbens, and amygdala, either directly or indirectly, acting in the interneurons. [1]

Identification of specific components of the basal forebrain that have been associated with drug reward have focused on the extended amygdala, which includes the central nucleus of the amygdala, the bed nucleus of the stria terminalis, and a transition zone in the medial (shell) part of the nucleus accumbens. The extended amygdala receives numerous afferents from limbic structures, such as the basolateral amygdala and hippocampus, and sends efferents to the medial part of the ventral pallidum and a large projection to the lateral hypothalamus, further defining the specific brain areas that interface classical limbic (emotional) structures with the extrapyramidal motor system. [1, 10]

The structures comprising the extended amygdala may further define the neuronal substrates for the acute reinforcing actions of drugs of abuse. Amygdala appears to act in accord with the ventral striatum (VS) to pick up stimuli that are not just emotionally salient but highly relevant to a task-dependent reward. [1, 11]

Neuroscience research has demonstrated a shared vulnerability in neuronal circuits that underlies the abuse of psychoactive substances toward delineating the neurobiological processes that constitute this vulnerability. Among those affected paths, we may detach dysregulation of mesolimbic DA circuits, reduction in DA D2 receptors (DRD2), abnormalities in the orbitofrontal cortex and the anterior cyngulate gyrus, anomalies in the ventromedial prefrontal cortex, differential genetic variants of cannabinoid receptor 1 (CB1/Cnr1) affecting its function, up-regulation of brain-derived neurotrophic factor (BDNF). [6]

The evidences gathered by the scientific researchers suggest that the recognizable behaviors that characterize the addiction phenotype (compulsive drug consumption, impaired self-control, and behavioral inflexibility) represent unbalanced interactions between complex networks (that form functional circuits) implicated in goal-directed behaviors.[12]

The ability of certain behavioral routines to become deeply ingrained, after enough repetition, helps to explain both the difficulty of suppressing them (i.e. compulsion) and the ease with which they bounce back after extinction (i.e. relapse). Habituation appears to be based mainly in the mesostriatocortical circuits that 're-code' the behavioral outcome of repetitive actions in a process that was aptly referred to as the 'chunking' of action repertoires. [12]

Drug-induced adaptations anywhere along this bidirectional circuitry, between VTA and the neighboring substantia nigra (SN), ventral and dorsal striatum, thalamus, amygdala, hippocampus, subthalamic nucleus, and the prefrontal cortex (PFC) can trigger or facilitate the addictive process by disrupting reward-based learning via the modulation of regional neuronal excitability**.** [12]

Many studies have established that DA signals emanating from the VTA/SN and arriving in the striatum, play a pivotal role in learning from past experience and orchestrating appropriate behavioral responses. Whether directly or indirectly, all addictive drugs have the power to cause large and transient increases in DA from VTA neurons that project primarily into the NAc of the ventral striatum (VS), but also to the dorsal striatum, amygdala, hippocampus and PFC. [12]

At the cellular and molecular level, genetic vulnerability to addictive drugs correlates, for example, with decreased neurofilamentary transport for tyrosine hydroxylase (the rate-limiting intraneuronal DA synthetic enzyme) in VTA/NAc DA reward-related neurons. This produces a DA deficiency in these VTA/NAc brain reward neurons, which is hypothesized to underlie vulnerability to addictive drug action. [6]

Another type of DA dysfunction in the VTA/NAc brain reward axis centers on a deficiency affecting DRD2 receptors. Blum and colleagues have long hypothesized that a deficit in normal DRD2 function in mesoaccumbens brain reward loci may confer vulnerability to drug addiction. [6]

3. Epidemiology and Etiology of Dependence

3.1 Epidemiological Facts

The costs of drug abuse and drug addiction to society are enormous in terms of both direct and indirect expenses associated with secondary medical events, social problems, and loss of productivity. In the United States alone, it is estimated that expenditure of illicit drug abuse and addiction is around $161 billion (Office of National Drug Control Policy, 2001). It is estimated that alcoholism costs to the society about $180 billion per year, and tobacco addiction require $155 billion (Centers for Disease Control and Prevention, 2004). In France, the total cost of drug use is $41 billion, including $22 billion for alcohol, $16 billion for tobacco, and nearly $3 billion for illicit drugs. [13]

Almost a quarter of the adult population in the European Union, corresponding to over 80 million adults, are estimated to have used illicit drugs at some point in their lives. Cannabis was the most popular (73.6 million users), with lower estimation reported for the lifetime use of cocaine (14.1 million users) (Figure 1). Levels of lifetime use vary considerably between countries, from around one-third of adults in Denmark, France and the United Kingdom, to less than 1:10 in Bulgaria, Greece, Cyprus, Hungary and Portugal. [13]

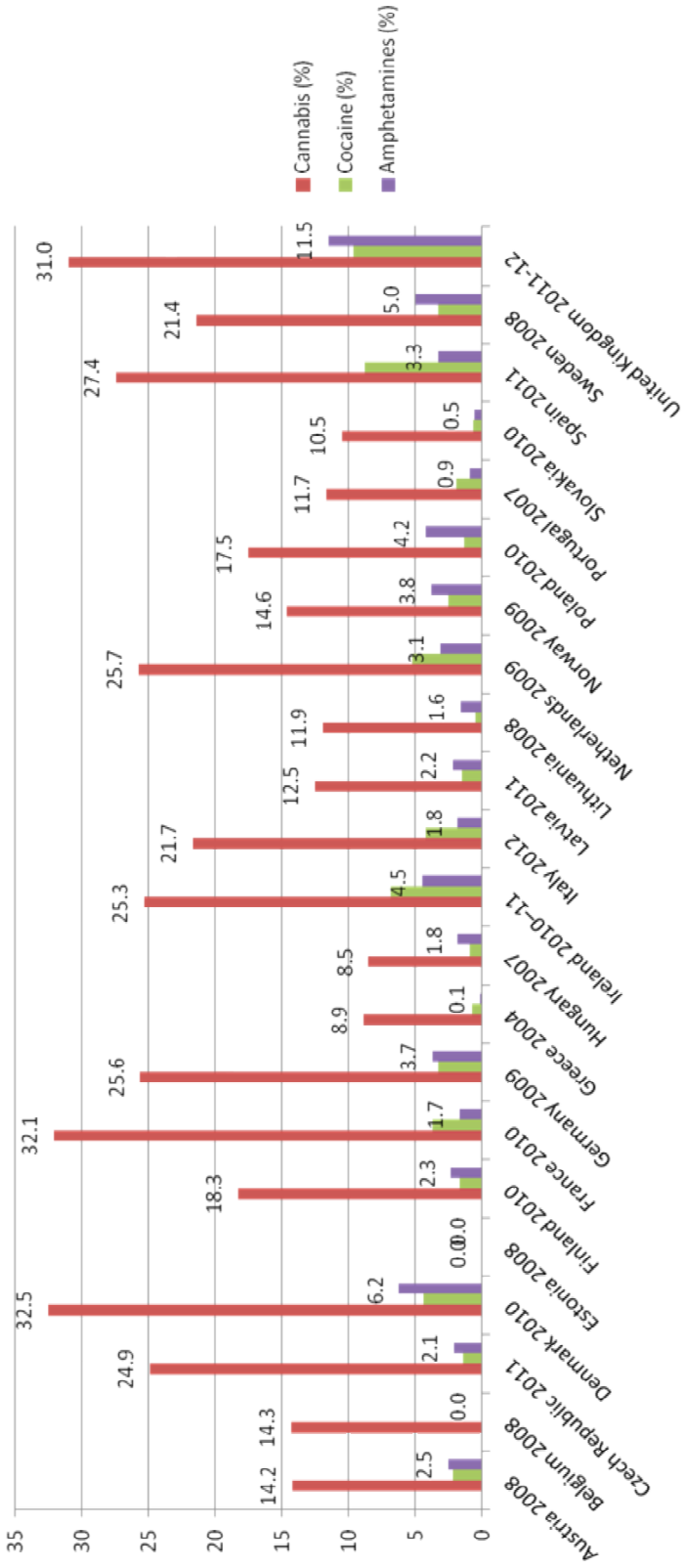

Figure 1: Lifetime prevalence of drug by European Union Countries (data available from http://www.emcdda.europa.eu/stats13, 2014)

Europe faces the dual challenge of developing effective responses to emerging problems and continuing to address the needs of drug users in long-term treatment. [13]

The bulk of costs related to treating drug use continue to stem from problems that are rooted in the heroin 'epidemics' of the 1980s and 1990s. Although initiation into heroin use may be in decline, heroin dependence, characterized by a chronic disease model with cycles of relapse and treatment entry, remains a key focus for interventions. The European Union has invested considerably high amounts of money in providing treatment opportunities for this group, currently with an estimation of three-quarters of a million in opioid substitution treatment. [13]

3.1.1- Opioid: Data of Consumption and Treatment

The illicit use of opioids remains responsible for a disproportionately large share of the morbidity and mortality resulting from drug use in Europe. The opioid most used in Europe is heroin, which may be smoked, snorted or injected. A range of other synthetic opioids, such as buprenorphine, methadone and fentanyl, are also available on the illicit market. Opioid use tends to be highest among marginalised populations in urban areas. [13]

The average annual prevalence of problems due to opioid use among adults (15–64) is estimated to be around 0.4 %, the equivalent of 1.3 million users in Europe in 2012 (Table 1). [13]

Table 1: Opioids estimate uses in European Union [13]

1.3 million users (15–64 years of age)
3.5% of all deaths of Europeans with 15–39 years old are due to drug overdoses, opioids are found in about three-quarters of fatal overdoses
Principal drug in about 45% of all drug treatment requests in the European Union
700,000 opioid users received substitution treatment in 2012

Addicted individuals using opioids, mainly heroin, as their primary drug, represent 46% of all drug users who entered specialized treatment during 2012, in Europe (180,000 subjects), and around 26% of those initiated treatment for the first time. [13]

In Figure 2, it is presented the data of drug users who entered treatment for the first time in 2011.

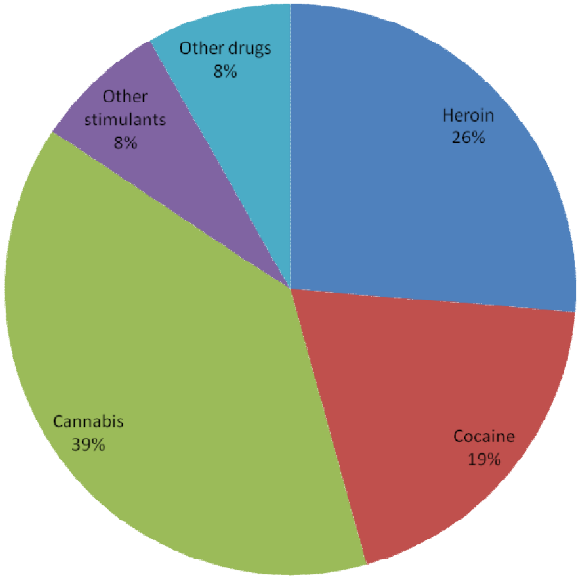

Figure 2: New addicted patients (%) entering treatment by primary drug heroin, cocaine, cannabis, other stimulants and other drugs during 2011 in Europe (data available from http://www.emcdda.europa.eu/stats13, 2014).

In 2012, in the majority of European countries, more than 10 % of first-time opioid users entering specialised treatment were misusing opioids other than heroin (Figure 3). These included methadone, buprenorphine and fentanyl. In some countries, these drugs now represent the most common form of opioid abusing use. [13]

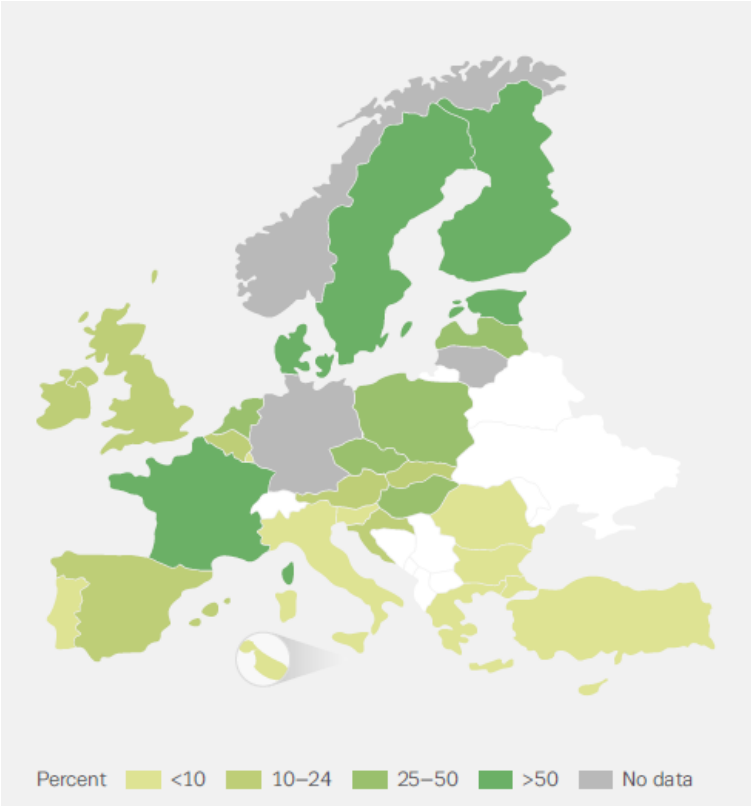

Figure 3: First-time entrants in treatment due to abuse of opioids other than heroin: trends in as percentage of all first-time entrants with opioids as primary drug. (data available from European Monitoring Centre for Drugs and Drug Addiction- **European Drug Report 2014: Trends and developments**. Luxembourg: Publications Office of the European Union, 2014)

While deaths related to heroin are generally falling, deaths related to synthetic opioids are increasing, and in some countries now exceed those attributed to heroin. Substitution treatment, typically combined with psychosocial interventions, is the most common treatment for opioids' dependence in Europe. The evidences available support this combined approach for keeping patients in treatment, as well as for reducing illicit opioid use, drug-related harms and mortality. [13]

3.1.2- Cocaine: Data of Consumption and Treatment

Cocaine powder is primarily sniffed or snorted, but is also sometimes injected, while crack cocaine is usually smoked. Among regular users, a broad distinction can be made between more socially integrated and moderate consumers, who may be using the drug in a recreational context, and more marginalised drug users, who use cocaine, often along with opioids, as part of a chronic drug problem. [13]

Cocaine is the most commonly used illicit stimulant drug in Europe (Table 1) but decreases in cocaine use are also observable in the most recent data. [13]

Table2: Cocaine estimate users in European Union [13]
14.1 million or 4.2 % of adults (15–64 years of age) used cocaine in their lifetime
3.1 million or 0.9 % of adults (15–64) used cocaine in the last year
2.2 million or 1.7 % of young adults (15–34) used cocaine in the last year
0.2 % and 3.6 % — lowest and highest national estimates of last year cocaine use among young adults

In Figure 4 it is presented data of last 12 months prevalence of cocaine abuse in several European Union countries, among all adults, young adults and youth.

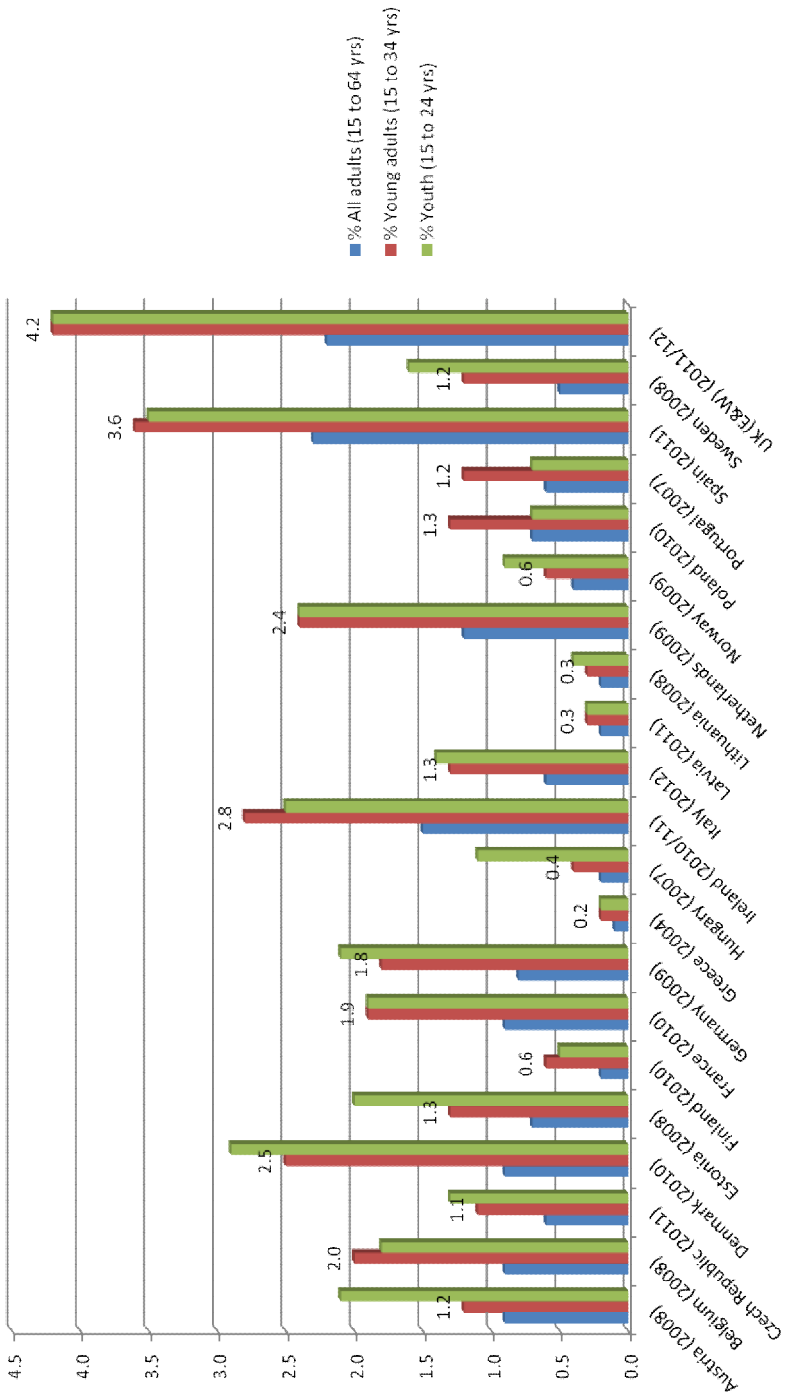

Figure 4: Last 12 months prevalence of cocaine use among all adults - aged 15–64, young adults - aged 15–34 and youth - aged 15–24 (data available from http://www.emcdda.europa.eu/stats13, 2014)

Cocaine was cited as the primary drug for 14% of all reported abusers entering specialised drug treatment in 2012 (55,000), and 18% of those entering treatment for the first time (26,000) (See Figure 2 for data of 2011). Differences exist between countries, with around 90% of all cocaine clients being reported by only five countries (Germany, Spain, Italy, Netherlands, United Kingdom). [13]

In 2012, around 77,000 cases of seizures due to cocaine were reported in the European Union, amounting to 71 tonnes of the drug being intercepted. The number of cocaine seizures reported in 2012 remains at a high level, compared to 2002. However, it has decreased from an estimated peak of around 95,000 seizures in 2008. [13]

Decreases in the quantity of cocaine apprehended are most observable in the Iberian Peninsula, particularly in Portugal between 2006 and 2007, and more gradually in Spain between 2006 and 2011. [13]

Survey data illustrate the geographical differences in stimulant use patterns in Europe. Cocaine is more prevalent in the south and west of Europe, amphetamines in central and northern countries, and ecstasy — albeit at low prevalence levels — in countries in the south and east, among young adults (Figure 2). [13]

Figure 5: Predominant stimulant drug by last year prevalence among young adults(15–34 years old) (data available from European Monitoring Centre for Drugs and Drug Addiction- **European Drug Report 2014: Trends and developments**. Luxembourg: Publications Office of the European Union, 2014).

3.2- Current Status in Portugal

In the study conducted in 2012 in the general Portuguese Population (15-64 years of age), cannabis, ecstasy and cocaine were the illicit substances preferably used by the Portuguese with lifetime prevalence (at least one use experience) respectively of 9.4%, 1.3% and 1.2%.
Between 2007 and 2012, in the set of the Portuguese population, it was verified for almost all drugs a decrease in lifetime prevalence (of any illicit drug from 12% to 9.5%) and recent use (of any illicit drug from 3.7% to 2.7%) as well as decrease in continuity rates of use (of any illicit drug from 31% to 28%). [14]
In 2012 was held in Portugal the III National Population Survey on Psychoactive Substances in the Portuguese Population (INPP – Inquérito Nacional ao Consumo de Substâncias Psicoactivas na População Portuguesa). [14]
In 2012, similarly to 2007 and 2001, cannabis was the illicit substance that registered the higher lifetime prevalence of use – at least one use experience in life – and recent use – in the last 12 months at the date of the enquiry, either in general population (15-64 years old) and in the young adult population (aged 15-34). These prevalences were, respectively, 9.4% and 2.7% in general population, and 14.4% and 5.1% in young adults. [14]
Comparatively to other European countries, with studies carried out between 2010 and 2012, and the same population age range (15-64 years) as reference, Portugal continues to present prevalence of use of illicit substances below the average values registered in those countries. [14]

3.3- Etiological Causes of Dependence

Environmental exposure, including social background and genetic factors contribute to individual differences in vulnerability to initiating use of addictive agents and in vulnerability to the shift from substance use to addiction. [15]
The addictions encompass also non-substance related behaviors, such as sexual, internet, gambling or food addiction, that are widespread and that might access the same neurobiological pathways that modulate reward, impulsive and compulsive behavior and mood. [15]
The origins of addiction vulnerability are complex and wide-ranging; the underlying genetic factors need to be identified to solve the puzzle of what causes the pervasive and relatively intractable disorders. [15]

Drugs differ in their addiction liability, which is the relative potential of an agent to lead to addiction. Cocaine and opiates, among the most addictive substances, are also among the most heritable, concerning family history. On the other hand, hallucinogens are among the least addictive, and are also the least heritable. These data seem to point towards an inheritance of variation in the core of neurobiological basis of addiction, such as the pathways that mediate reward, behavioral control, compulsivity, or stress and anxiety response. [15]

Addictions are inherited as common, complex diseases that show no obvious pattern of Mendelian transmission, but with evident genetic involvement and heritability. The identification of specific genes and functional loci moderating vulnerability has been challenging because of the genetic complexity of addictive disorders, namely related with underlying neurobiological pathways. This complexity derives from multiple sources including incomplete penetrance, phenocopies, variable expressivity, gene-environment interactions, polygenicty, genetic heterogeneity, among others. [5]

It is tempting to imagine that addictions are polygenic, with vulnerability arising from the simultaneous action of functional variations at multiple genes. However the complexity of neurocircuitry and neurobiology can lead to highly intricate genetic heterogeinity, meaning that a single genetic variation can determine vulnerability, but different variants can be enough for expression of the disease in different individuals and families. [15]

One strategy to discover gene effects in etiologically complex diseases, such as addiction, is the deconstruction of phenotypes into elements that are etiologically less complex. Intermediate phenotypes access mediating mechanisms of genetic and environmental influences. These heritable intermediate phenotypes are endophenotypes. For example, alcohol-induced flushing is a protective alcohol-related endophenotype, influenced by alleles mediating variation in alcohol metabolism and low response to alcohol has been associated with genetic variation, namely in the serotonin transporter gene (SLC6A4) and in the gene encoding the subunit a6 of the γ-aminobutyric acid receptor A (GABRA6). [5]

4. Tools for Identification of Genetic Markers

A first step must be given to evaluate the evidence concerning the extent to which substance abuse disorders may be influenced by heritable factors. [16]

There are two main types of studies, linkage and association studies, conducted to establish whether genes and their variants may be involved in causing or in vulnerability to drug addiction. [16, 17]

Linkage studies use families to provide evidence of how close a genetic marker is to an allele causing the phenotype under study, whereas association studies may be performed with unrelated individuals. Strong evidences can be derived from a range of family-based genetically informative research designs including family, adoption and twin studies. [16, 17]

Early family-based studies provided initial clues of potential heritable influences by examining the risk of substance use addictive disorders in the first-degree relatives of individuals either with or without a substance use disorder. [16, 17]

The classical twin study design makes use of data from monozygotic (MZ) and dizygotic (DZ) twin pairs, reared together, to attempt to disentangle the role of genetic and environmental influences on population variation in a measurable phenotype. Genetic variants are shared completely between members of MZ twin pairs while DZ pairs share on average 50% of their genetic variants. In any case this can only be a starting point, as a demonstration that there are heritable factors that influence individual differences in vulnerability to addiction. [16]

Case control-association studies are another approach to identify variants involved in addictions. It consists of selecting genes that are likely to be involved in the physiological effect of the specific drug under consideration in a neurotransmitter system (receptors, transporter, metabolizing enzymes, etc), related to drug taking behaviors. Genetic variants are identified in these candidate genes. Cases and controls are genotyped for the variants and statistical analyses are then applied to evaluate the probability that a given variant allele is associated with the drug addiction. [17]

In association studies, the ethnicity of the subjects must be carefully evaluated because some genes' allelic frequencies vary widely among ethnic groups. If this issue is not addressed, there are bias that compromise interpretation of results. [17]

The techniques for conducting association studies have seriously improved once thousands of variants can be included using gene array technology. To locate and identify genes and chromosomal regions that are associated with specific addictions, genome-wide scans can be

performed on affected and control subjects populations, in order to identify significant differences in frequency distribution, allowing to point candidates contribution for risk or vulnerability to addiction. [17]

Candidate gene approaches have tended to focus upon specific sets of genes based on assumptions about the importance of certain genes in addiction, usually assessing a small number of genomic markers. These assumptions were based mainly in the mechanisms of action of particular drugs of abuse, e.g. dopamine systems (cocaine and other stimulants), opioid systems (heroin and other opiates), GABAergic systems (ethanol), among others. [16]

Candidate gene and genome-wide analyses have been increasingly integrated in research, in order to identify genetic variations influencing addiction. [5]

Genome-wide association studies measures and analyzes DNA sequence variations from across the human genome in an effort to identify multiple genetic risk factors for diseases that are common in the population under study. [19]

A few concepts must be clarified before carrying on with the analysis of methodology to discover genetic variations. One of these concepts is the common disease/ common variant (CD/ CV) hypothesis. This hypothesis simply states that common disorders are likely influenced by genetic variation that is also common in the population. Another important concept is linkage disequilibrium (LD). LD is a property of SNPs on a contiguous stretch of genomic sequence that describes the degree to which one allele of a SNP is inherited or correlated with an allele of another SNP within a population. LD is a terms used to designate for the chance of co-inheritance of alleles at different loci. LD is due to recent migration, selection or recent mutation. The SNPs that are specifically selected to capture the variation at nearby sites in the genome are called tag SNPs, because alleles for these SNPs tag the surrounding stretch of LD. Patterns of LD are population specific and as such, tag SNPs selected for one population may not work well for other populations. [19, 20]

Genome-wide association studies were made possible by the availability of chip-based microarray technology for assaying more than one million SNPs at once. Two primary platforms have been used for most commonly used GWAS. These include products from Illumina (San Diego, CA) and Affymetrix (Santa Clara, CA). The Affymetrix platform prints short DNA sequences as a spot on the chip that recognizes a specific SNP allele. Alleles are detected by differential hybridization of the target DNA sample. On the other, hand Illumina uses a bead-based techonology with slightly longer DNA sequences to detect alleles. [19]

As compared to candidate gene investigation, genome-wide association studies (GWAS) have the advantage of covering the entire genome in an hypothesis-free way and it is a powerful method to detect relatively common alleles of moderate effect. [5]

Another advantage of GWAS is related to the fact that the same genotyping arrays are obtained in different samples facilitating the comparison of results from different studies in meta-analyses. This is a crucial characteristic, because extremely large numbers of samples are required, in order to detect the small effects of many common variant on complex diseases. It is worth to notice that in GWAS, up to 5 million SNPs can be simultaneously tested raising the issue of false positive due to multiple testing. For achieving an effective *p* value of 0.05 the genome-wide significance threshold is usually set at approximately 10^{-8}. [5]

GWAS for addiction is in a relatively early stage, with missing of several addictions to be evaluated and the number of samples that have been studied so far have either not been very large (<10,000) or have been flawed by cross-country heterogeneity, having less than optimal phenotying and insufficient number of subjects with an extreme phenotype. [5]

So far, the strongest and confirmed *locus* detected by GWAS is for the CHRNA5-CHRNA3-CHRNB4 gene cluster on chromosome 15q25. This region harbors a locus-altering tendency to nicotine addiction. Association of genetic variation, within this region, to smoking behavior was initially discovered using a candidate gene approach but was subsequently replicated by GWAS. [5]

Since the development of high-density microarray technology, it has become possible to identify a large number of single nucleotide genetic variants in a single individual. Statistical analyses comparing groups of individuals using these high-density microarrays have allowed researchers to conduct genome-wide association studies. These studies have provided confirmatory evidence for the involvement of previously identified genetic variants and the genes containing these variants, as well as evidence for the involvement of genes and genomic regions that have not been previously associated with addictions**.** [18]

However, the impact of less common variants cannot be assessed by using the current GWAS arrays and requires sequencing strategies. It is important to note that the technology for finding genomic variations is changing rapidly. Chip-based genotyping platforms, such as those mentioned above, will be most probably replaced over the next years with new technologies for sequencing the entire genome. These next-generation sequencing methods will provide all the DNA sequence variation in the human genome. [19]

Since the completion of the first human genome sequence in 2003, demand for cheaper and faster sequencing methods has markedly increased. This demand led to the development of next-generation sequencing (NGS). [18]

NGS data output has increased since it was invented. In 2007, a single sequencing run could produce a maximum of around one gigabase (Gb) of data and by 2011 that rate had nearly reached a terabase (Tb). [21, 22]

During the past decade, several platforms have been developed. NGS platforms perform massively parallel sequencing and this technology facilitates high-throughput sequencing, which allows an entire genome to be sequenced in less than one week. However, data analysis may take several weeks, for analyzing the variants in 3 billion base pairs and its significance is sometimes difficult to establish. [18]

5. Dependence and Genetic Factors

5.1 Genetic markers of alcohol addiction

Alcohol dependence is characterized by a cluster of cognitive, behavioral, and physiologic symptoms, with and affected individual continuing to drink, despite significant alcohol-induced impairment or distress**.** [23]

Alcohol drinking is highly prevalent in many cultures and contributes to the global burden of disease. In fact, it is linked to more than 60 diseases, including cancers, cardiovascular diseases, liver cirrhosis and neuropsychiatric disorders and the World Health Organization estimates that approximately 76.3 million people have alcohol-linked disorders, besides car accidents in consequence of alcohol abuse. [24, 25]

On a population basis, alcoholism alone subtracts an average of 4.2 disability adjusted life years (DALYs), which are the years of life that are lost due to premature mortality or disability, per person; tobacco subtracts 4.1 DALYs and illicit drugs subtract 0.8 DALYs. For comparison, AIDS subtracts 6.0 DALYs and type 1 diabetes subtracts 0.1 DALYs. [15]

Alcohol shares in common with nicotine, cocaine, amphetamine, heroin and morphine, the property of enhancing dopamergic transmission in ventral striatum and medial prefrontal cortex. This release of dopamine is partially enhanced by stimulation of μ-opioid receptors (for which endorphin is the primary ligand) located on inhibitory GABAergic interneurons in the VTA. [26]

The GABAergic interneurons inhibit the dopaminergic ventral tegmental neurons, whose activation signals the reward launch. Thus, μ-opioid receptor agonists enhance the likelihood of ventral tegmental dopaminergic neuron activation (and the experience of reward) by lessening the tonic inhibition of the associated GABAergic interneurons. [26]

Endorphin elevations after alcohol intake are seen in discrete reward regions of the hypothalamus, ventral tegmentum, and ventral striatum. [26]

In the 1980s, a substantial evidence was developed that naltrexone, an orally active μ-opiod receptor antagonist, could decrease alcohol self-administration, craving and relapse to heavy drinking, but did not reduce abstinence rates. There have been more than 30 clinical trials of naltrexone in alcohol addiction showing the efficacy of naltrexone in reducing risk for relapse to heavy drinking, although the effect size is small, with many patients having no benefit. These results confirmed the neurobiological effects of alcohol in reward circuits related to opioids. [26]

A meta analysis, which included 9,987 monozygotic and dizygotic twin pairs, estimated a heritability of alcoholism to lie around 50-60%. [15]

Researchers have identified several genes that predispose individuals to developing alcohol dependency and encode for proteins that play a role in the pharmacokinetics and pharmacodynamics of ethanol; alterations in these factors alter the rewarding effects of alcohol, thereby affecting its abuse liability [25]

A common missense single nucleotide polymorphism (rs#1799971) in the first exon of the μ-opioid receptor gene, *OPRM1*, was described in 1997, c.118A>G, or p.N40G, reflecting the fact that the A allele encodes asparagine, whereas the minor G allele encodes aspartate. Subsequent studies revealed large ethnic differences in allele frequencies (see Table 3). [26]

Table 3: Frequency of G allele for c.118A>G SNP in ethnic groups (adapted from Berretini, 2013)

Ethnic Group	Frequency of G Allele
African	1%
African-American	3%
European-American	15%
Chinese	35%

Many studies have been carried out in order to understand the functional consequences of this allelic variant. Beyer et al. (2004) reported that the 118G allele was not different from the 118A allele in rate of internalization, but 118G had decreased transcription, compared to 118A. Similar results were obtained by Zhang et al. (2005) that revealed a marked decrease in 118G allele mRNA. [26]

In a laboratory investigation of c.118A>G pharmacogenetics, related to alcohol reward in humans, Ray and Hutchison (2004, 2007) showed that the G allele carriers experienced significantly stronger euphoria sensation after standard oral doses of alcohol, compared to AA individuals. In agreement with this result, Ramchandani reported that G allele carriers had a higher striatal release of dopamine after alcohol (using detection of raclopride binding to DA receptors by PET scan), compared to AA participants. These laboratory studies of the human c.118A>G variant on effect of alcohol are remarkably consistent, with the clear

conclusion that the G allele allows people to experience alcohol in a more rewarding manner, compared to AA individuals. [26]

Multiple neurotransmitters are involved in orchestrating ethanol's reward profile, including dopamine, γ–aminobutyric acid (GABA), glutamate, and serotonin. Ethanol has been shown to enhance the function of γ–aminobutyric acid receptor type A (GABAA), neuronal α2β4 nicotinic acetylcholine, and glycine receptors, and to inhibit N-methyl-d-aspartate-(NMDA) type glutamate receptor function. The polymorphism c.1236C>T, in the gene that encodes the α6 subunit of the GABAA receptor, has been associated with a low level of response to alcohol, which is a strong predictor of developing alcohol dependency. Other SNP haplotypes in the gene encoding the α1 subunit have also been detected at higher frequencies in alcohol-dependent individuals. [25]

Alcohol dehydrogenase (ADH) metabolizes ethanol to acetaldehyde, a toxic intermediate, which is then metabolized to acetate by aldehyde dehydrogenase (ALDH). Variations in the genes encoding these enzymes can alter alcohol metabolism and result in the accumulation of acetaldehyde during alcohol consumption, which causes a flushing response as well as headache, nausea, and palpitations. Functional polymorphisms in multiple alcohol dehydrogenase coding genes, namely *ADH4*, *ADHIB*, and *ADHIC*, as well as the aldehyde dehydrogenase coding gene *ALDH2*, have been shown to alter the risk for developing alcohol dependency. Twelve SNPs in and around the *ADH4* gene have been consistently associated with a higher risk for alcohol dependency in a variety of populations. In particular, the -136C>A polymorphism in the promoter region of *ADH4* has been extensively studied. The -136A allele has been associated with an increased susceptibility to alcohol dependency. The -136A allele, associated to higher activity, caused a lower peak blood ethanol level after alcohol ingestion, compared to the -136C allele. The *ADH1B**2 variant was associated with increased ethanol oxidation to acetaldehyde and has been shown to protect against alcohol dependency in a variety of populations. [25]

The enzyme Cytochrome P450 2E1 (CYP2E1) also participates to the metabolism of ethanol, mainly in liver. CYP2E1 accounts for approximately 20% of ethanol metabolism at low blood concentrations, and its contribution increases to 60% at high concentrations. *CYP2E1*1D* has been shown to have increased enzymatic activity and has been associated with alcohol dependency, although inconsistent results have been obtained across studies. The *CYP2E1*5B* polymorphism has been associated with altered transcriptional activity of the

CYP2E1 gene. CYP2E1*5B has been associated with higher ethanol consumption and risk for alcohol dependency. [25]

Twin and adoption studies suggest that familial pattern of alcohol dependence may be attributable to additional genetic factors, which account for roughly 40-60% of the liability for this addiction. [27]

Due to the etiological complexity of complex traits like alcohol dependence, newer DNA sequencing methods, in particular, next generation sequencing (NGS) have become increasingly useful as they provide a more accurate description of both common and rare variants. So far, molecular genetic studies have linked variations across chromosomes 1, 2, 3, 4, 7, and 8 to diagnoses of alcohol dependence, as well as chromosomes 5, 6, 9, 15, 16, and 21, using quantitative phenotypes (e.g., maximum number of drinks) and neurophysiological phenotypes that are often comorbid with alcohol dependence and other psychiatric disorders. To date, at least one variant in about 602 genes has been linked to alcoholism and/or alcohol dependence. [27]

Pharmacogenetic research has also shown that variations within *ADH* and *ALDH* genes may alter a person's risk for developing alcohol-linked problems. For example, of the seven genes that code for different forms of ADH (clustered on chromosome 4q), variants within the genes encoding the hepatic isoforms, *ADH1B* and *ADH1C* have been related to alcohol dependence. The *ADH1B*2* allele has also been shown to have a protective role against alcoholism in males and females of different ethnic origins. [27]

Concerning ADH, three class I isoenzymes are known for their genes closely linked, located in chromosome 4q22. Three different alleles, *ADH1B*2*, *ADH1B*3* and *ADH1C*1*, have been shown to alter ADH enzymatic activity, with *ADH1B*2* and *ADH1B*3* increasing the activity more than 30-fold. As individuals carrying these alleles are likely to have a higher concentration of acetaldehyde, a hypothetical protective effect can be proposed. The allele *ADH1C*1* is found with a frequency of 55–60% in Europeans. A meta-analysis revealed that *ADH1B*2* has protective properties decreasing the risk of alcoholism by a factor of 3, compared to the *ADH1B*1* allele. [28]

As a consequence of genotyping 110 SNPs throughout the *ADH* gene cluster, located on chromosome 4, the results revealed twelve SNPs in *ADH4* gene and sorroundings that were significantly associated with alcoholism. It was also shown that there was a modest evidence of association with SNPs in *ADH1A* and *ADH1B*, suggesting that alleles of these genes contribute to alcoholism susceptibility. From nine gene families encoding for human ALDH, only ALDH1 and ALDH2 are centrally involved in the oxidation of acetaldehyde; ALDH2

plays the major role in the acetaldehyde oxidation. The *ALDH2*2* allele is associated with enzyme inactivity resulting in symptoms of acetaldehyde syndrome. Therefore, ALDH2*2 seem to reduce the risk of becoming alcohol dependent by 10-fold, thus providing a stronger protective effect as compared to alterations reported in *ADH1B* and *ADH1C* genes. [28]

Approximately 45% of East Asians (Japanese, Chinese, Koreans) are carriers of the *ALDH2*2* allele (Glu504Lys, rs#671) that leads to the inactive ALDH2 enzyme. After consumption of small quantities of alcohol by these individuals, the endotoxin acetaldehyde rapidly accumulates, resulting in the very unpleasant flushing syndrome (facial flushing, tachycardia, sweating, headaches, nausea), colloquially called 'Asian Glow' or 'Asian Blush', that is protective against heavy drinking and therefore alcoholism, related to the unpleasant physiological features mediated by acetaldehyde. [29]

The two examples of verified human "addiction genes" encode for enzymes that catalyse consecutive steps in alcohol metabolism, as ADHIB and ALDH2. The most important loci at these genes are p.His47Arg in the *ADH1B* gene and p.Glu487Lys in the *ALDH2* gene. Either higher activity of ADH1B (conferred by the His47 allele) or lower activity of ALDH2 (conferred by the Lys487 allele) leads to accumulation of acetaldehyde after alcohol consumption, which causes the aversive flushing reaction described above. The genotype-associated flushing is equivalent to the effects of disulfiram that inhibit ALDH. In several eastern countries, such as Japan, where both His47 and Lys487 are highly abundant, most of the population carries a genotype that is protective against alcoholism. [15]

Table 4: Evidence of the genetic influence on alcohol consumption from genetic studies: summary of results of ADH genes (adapted from Köhnke (2008))

Examined Polymorphism/ Chromosomal region	Examined phenotype	Method	Reference
*ADH1B*2*	Protective against alcohol dependence	Case control	Shen YC, 1997
*ADH1B*2*	Protective against alcohol dependence	Meta-analysis	Whitfield JB, 1998
*ADH1B*2*	Reduced level of weekly peak alcohol intake	Case control	Neumark YD, 1998
*ADH1B*2*	Protection against alcohol related birth defects	Case control	Viljoen DL, 2001
*ADH1B*3*	Protection against alcohol related birth defects	Case control	McCarver DG, 1997
*ADH1B*3*	Negative family history of alcoholism	Case control	Ehlers CL, 2001
*ADH1B*3*	Protective against alcohol dependence	Linkage analysis	Edenberg HJ, 2006
*ADH1C*2*	Protective against alcohol dependence	Case control	Shen YC, 1997
ADH4 AC	Alcohol dependence	Case control	Guindalini C, 2005
ADH4 SNP2, SNP3	Alcohol dependence	Family-based association	Luo X, 2005
SNPs: chromosomal region 4q21-23	Alcohol dependence	Linkage analysis	Reich T, 1998; Long JC, 1998; Prescott CA, 2006; Edenberg HJ, 2006

GABA is the major inhibitory neurotransmitter in the central nervous system. GABA A receptors are sensitive to ethanol in distinct brain regions and are clearly involved in acute actions of ethanol, ethanol tolerance and ethanol dependence. [28]

A linkage study revealed evidence of linkage to a chromosomal region on chromosome 4p near the β1 GABA receptor gene (GABRB1), a finding that was confirmed by an association study which revealed a significant association between GABRB1 and alcoholism. [28]

A German consortium, *Genetics of Alcohol Addiction*, which aims to identify and validate candidate genes and molecular networks involved in the aetiology of this pathology, published the first GWAS for alcohol dependence. One goal of this systems genomic approach is to provide an understanding of the complex mapping relationship between the genome and disease, by investigating intermediate endophenotypes. By genotyping a dense set of SNPs throughout the genome, researchers have the potencial to identify with considerable precision genes involved in alcohol dependence. [24, 30]

This GWAS was performed in 487 patients and 1,358 controls, allowing identification of 121 SNPs with nominal $p<0.0001$ and these SNPs were genotyped in the follow-up sample. As a result, fifteen SNPs showed significant association with the same allele as in the GWAS. In the combined analysis, two closely linked SNPs in the 3´flanking region of the peroxisomal trans-2-enoyl-coA reductase (PECR) gene, achieved genome-wide significance (rs#7590720; rs#1344694). [24]

Treutlein and colleagues (2009) conducted a GWAS and follow-up study for alcohol dependence using individual genotyping in a German male sample. In total, 139 SNPs were carried forward for genotyping in the follow-up study and three genes were confirmed to be associated to alcohol consumption: alcohol dehydrogenase 1c (ADH), cadherin 13 (CDH) and gata-binding protein 4 (GATA). Two markers, rs#7590720 and rs#1344694, remained significant after genome-wide correction in the combined sample of 1,460 patients. The two markers are located approximately 5 kb apart in chromosome region 2q35, which has been implicated in linkage studies for alcohol dependence phenotypes. Linkage to this region was found in a genome-wide search in 2,282 individuals from 262 families with a high prevalence of alcohol dependence in the Collaborative Study on the Genetics of Alcoholism (COGA). [23]

Candidate gene strategies have identified significant associations between SNPs in the gene encoding the alpha2-subunit of the γ-aminobutiric acid A receptor (*GABRA2*) and there are multiple positive reports of association between SNPs in *GABRA2* and alcohol abuse phenotypes. [30]

Bierut et al. (2010) reported on a large sample of 1,897 alcohol dependent cases from the "Study of Addiction" (Genetics and Environment analysis of 948,658 SNPs that span the genome). Primary analysis from GWAS identified 15 SNPs with $p<10^{-5}$ and the top associated SNPs were tested for replication in two independent datasets. The first replication sample is the family-based study from the Collaborative Study on the Genetics of Alcoholism (COGA) and none of the SNPs showed association with a $p<0.05$; however, rs#1386449 and rs#10224675, which in the primary analysis were associated with alcohol dependence only in African-Americans have a $p<0.10$ in the family based analysis with a small number of African-American families. Of the seven SNPs that were genotyped, for none a significance level of $p<0.05$ was reached. [30]

Only one SNP (rs#13160562) shows modest evidence of replication for SNPs reported in the independent GWAS of alcohol-dependent men by Treutlein et al. (2009). In a meta-analysis, this SNP did not reach genome-wide significance (OR= 0.83, 95% CI 0.77-0.90, $p=2.74x10^{-6}$). [30]

This analysis confirms the modest association of alcohol dependence with variants in *GABRA2*. The two genome-wide significant results reported by Treutlein and colleagues, rs#7590720 and rs#1344694, were not replicated in this study. [30]

In addition to the possibility that some of the top signals were false positives, the high levels of comorbid substance-use disorders may have increased the odds to identify association to genes contributing to addiction in general and may potentially limit the ability to replicate these association results in samples ascertained solely for alcohol dependence. [30]

Advantages of the genome-wide design include its hypothesis-free strategy and its suitability for the discovery of novel genetic contributors to disease. However, the genome-wide examination requires correction for multiple testing and the threshold for significance of GWAS findings is high. On the contrary, targeted gene studies test specific hypothesis to provide validation of previously reported findings and require much lower threshold for significance. [30]

To increase the power to detect significant results, two strategies can be taken: enlarge the sample or to narrow the phenotype to increase the detectable genetic effect. [30]

Gelernter reported a GWAS of alcohol dependence in European-American (EA) and African-American (AA) populations, with a total sample of 16,087 subjects. GWAS was used in order to identify genetic variants that influence risk of AD as both a diagnosis and an ordinal trait in EA and AA subjects. In ordinal trait analysis, the genome-wide association results for numerous variants were significant. The highest number of significant findings map

to the region of the ADH gene cluster in chromosome 4. There is a strong evidence for association with *LOC100507053* (a l*ncRNA* gene) and *ADH1B* in both AAs and EAs, most notably *ADH1B* SNPs rs#1229984 ($p=1.14x10^{-6}$) in EAs and rs#2066702 ($p=3.20x10^{-5}$) in AAs. In EAs, the association with rs#1229984 was observed previously by Bierut et al. (2010), but with a much weaker significance. The finding with rs#1789882 is the first GWAS finding for alcohol dependence in AAs, although the risk locus was known previously. [31]

In their study, Gelernter et al. (2014) identified a novel genome wide association ($p=5.57x10^{-10}$) with rs#1437396, which is located between and within 10kb of *MTIF2* (mitochondrial translational initiation factor 2) and *CCDC88A* (coiled-coil domain containing 88A) on chromosome 2, a risk locus that was supported by evidence obtained from analysis of both the EA and AA samples. It interacts with *DISC1*, a gene originally known to be as a schizophrenia risk locus, but association with opioid dependence has also been show. [31]

The GWAS approach to alcohol dependence suggests that this a genetically heterogeneous dependence; however, some candidate genes have been validated, as the *ADH1C* gene. GWAS of alcohol dependence and related phenotypes have identified numerous loci, but these loci alone have limited usefulness, once each one accounts for less than 1% of the variance in liability. Despite this alcohol GWAS continue to be studied because it support pathways that were hypothesized from linkage study findings and also highlight pathways that were not initially considered. [27]

Several new challenges arise from the fact that hundreds of genetic variants, each with a modest effect, contribute to its liability. Challenges, such as the identification and selection of polymorphisms, the reduction of the heterogeneity of alcohol phenotypes and the development/ implementation of the mathematical approaches and a conceptual framework will provide power and a meaningful interpretation of the findings. [27]

Missing heritability in GWAS has been attributed to the emphasis on common genetic variants that have low penetrance. As the number of variants tested on GWAS evolved from testing thousands of variants to more than 1 million, the likelihood of capturing variants that are in linkage disequilibrium with rare variants has increased. These observations suggest that when treated individually, common variants, such as SNPs, account for a small fraction of the missing heritability. The most likely solution to this problem would be the incorporation of both common and rare genetic variants using whole genome sequencing platforms. [27]

Missing heritability of GWAS in alcohol dependence can also be attributable to the fact that the liability of the disease is genetically and phenotypically heterogeneous. This reflects the

fact that people become addicted for different reasons. The lack of power of GWAS in alcohol dependence may be explained by the use of phenotypes that fail to capture the biological underpinnings of the pathology, which would lead to the classification of groups of alcoholics that may be more genetically homogenous. [27]

Alcohol dependence genetic complexity highlights the need for comprehensive models. System-based genetic studies of AD have become increasingly possible because of the major advances in genomics, proteomics, gene *vs*. environment interaction and correlation studies, and epigenetics. The combination of DNA whole genome genetic variations, with epigenetic, transcriptomic and proteomic profiles, taken from selected neuronal tissues involved in different stages of addiction, would be the ideal approach to achieving systems-based models for alcohol dependence. [27]

5.2 - Genetic markers of cocaine addicition

Cocaine is a central nervous system stimulant that acts primarily at the dopamine transporter DAT1, preventing dopamine uptake into presynaptic terminals and increasing synaptic dopamine levels. The susceptibility to cocaine dependency has been associated with variations in the genes involved in monoaminergic transmission. [25]

The profound loss of behavioral control is the Hallmark of cocaine addiction and contributes to the high risk of relapse. [33]

The psychostimulant properties of cocaine stem from its ability to inhibit reuptake by DAT1, but also acts at serotonin, and norepinephrine transporters, leading to the increase in neurotransmitters' synaptic levels. [34]

While the interplay between genetic and environmental factors underlying cocaine dependence is not fully understood, several studies have estimated that approximately two thirds of an individual's risk for developing this addiction is heritable. [33]

Identifying genetic risk factors is difficult due to the complex mode of inheritance, as well as clinical and genetic heterogeneity of cocaine-dependent individuals and strong environmental influences. Furthermore, associated genetic variations may be only a small contribute to the overall risk. Twin and family studies have demonstrated that cocaine addiction has a strong genetic component but the exact basis of the heritable factors that have a significant contribution to this phenotype remains unclear. [33, 35]

Genes involved in dopamine neurotransmission are biologically plausible candidate genes for cocaine addiction, since dopamine pathways play a major role in drug reward effect. Specifically, genes for dopamine receptors and transporters are logical targets for study, since they are directly responsible for transmitting dopamine-mediated brain signals. [33]

Cocaine addiction is accompanied by a decrease in striatal dopamine signaling, measured as a decrease in DRD2 binding as well as blunted dopamine release in the striatum. These alterations in dopamine neurotransmission have clinical relevance, and have been shown to correlate with cocaine-seeking behavior, as well as with response to treatment for cocaine dependence. [36]

The *DRD2* gene encodes an inhibitory dopamine receptor subtype. The striatopallidal medium spiny neurons, the cells involved in psychostimulant reward pathways, predominantly express this dopamine receptor subtype. Hence, variations in the *DRD2* gene may affect dopamine signaling via the striatopallidal pathway and, consequently, increase susceptibility to addiction by cocaine. While many single nucleotide polymorphisms (SNPs) spanning in the *DRD2* gene are cataloged, such as the TaqIA SNP (rs#1800497) has been shown to affect directly dopamine binding with DRD2. Furthermore, this polymorphism has been previously implicated in drug addictions such as heroin dependence and alcoholism. Therefore, the TaqI A SNP in the *DRD2* gene is probably a biologically functional candidate variant underlying susceptibility to cocaine dependence. [33]

Another plausible susceptibility gene for C is *SLC6A3*, coding for the dopamine transporter gene DAT1. The DAT1 protein mediates the active dopamine reuptake from the synaptic cleft into the presynaptic terminals, regulating the duration and intensity of dopaminergic signaling. Cocaine's pleasurable and addictive effects are thought to be mainly mediated through the blockage of DAT1, substantially increasing the concentration of extracellular DA, resulting in elevated stimulation of neurons involved in reward and reinforcement behavior. [33, 37]

Like DRD2, DAT1 is expressed in the striatal neuroanatomical region, which is implicated in cocaine reward. Many polymorphisms across the *SLC6A3* gene have been identified. The variable number tandem repeat (VNTR) polymorphism in the 3' region of *SLC6A3*, consists of a 40-bp repetitive sequence, which results in lower expression of the dopamine transporter in the putamen. It has been reported that the 10-repeat allele (10R) enhances the expression of the DAT1 protein while another study claimed that the 9- repeat allele (9R) enhanced the *SLC6A3* transcription and DAT1 expression. Although the specific results of each study conflicted, both reports suggest that the *SLC6A*3'_VNTR polymorphism affects

DAT1 expression, consistent with subsequent findings that this VNTR is associated with drug addictions such as methamphetamines and alcoholism. [25, 33]

A 40-bp VNTR in the 3' terminal, which results in lower expression of the dopamine transporter in the putamen, was shown to affect a variety of smoking behaviors and the risk for cocaine-induced paranoia. [25]

Both *DRD2* and *SLC6A3* have been investigated in cocaine addiction and positive associations have been found among Caucasian European and Brazilian populations. [33]

A brazilian study examined the functional influence of genetic *SLC6A3* variants on DAT1 expression, related to cocaine addiction and repeat polymorphisms were genotyped in cocaine-dependent abusers (n=699), including a 30-bp VNTR in intron 8 (Int8 VNTR). Their results revealed that the 3' UTR VNTR is not unique and there are approximately 15 other candidate simple tandem repeats and VNTRs in the introns of *SLC6A3* with at least six copies. Guindalini et al. (2006) identified a positive association between the 30-bp VNTR in Int8 of the DAT1 and cocaine abuse. [37]

Another study in a Spanish sample (n=169) that aimed to analyze several polymorphisms in *SLC6A3* (VNTRs in the 3' untranslated region, 3'UTR, and in intron 8), *DRD2* (TaqIA and TaqIB SNPs in 3'UTR and in intron 1) and in the gene coding for one enzyme of dopamine biosynthesis, DA beta-hydroxylase, *DBH* (19-bp insertion/deletion in 5'UTR and c.444G>A in exon 2) showed no significant association was found between cocaine dependence and the 3'UTR VNTR of DAT1, the TaqIA and TaqIB of DRD2 and the 19-bp insertion/ deletion and c.444G>A of DBH. Despite these results, a nominal association between cocaine dependence and the 5R/5R genotype of the Int8 VNTR within the DAT1 gene was found. [38]

These conflicting results highlight the need for more extensive association studies in terms of sample size and genetic coverage. [38]

The enzyme DBH catalyzes the conversion of dopamine to norepinephrine (NE) and could, therefore, have an influence on both cocaine action and the basal sensitivity of neurotransmitter systems to cocaine. It has been demonstrated that *DBH* knockout mice are hypersensitive to the psychomotor, rewarding, and aversive effects of cocaine. Pharmacological treatment studies with the DBH inhibitor disulfiram also indicate that this medication has efficacy as a treatment for cocaine dependence. [39]

DBH plasma activity levels were reported to vary widely among individuals. Cubells and colleagues (2000) found that a 19-bp insertion/ deletion polymorphism and the SNP

c.444A>G were associated with plasma DBH levels and that alleles of similar results for association to enzymatic levels were in significant positive disequilibrium. [40-42]

Guindalini et al. (2008) conducted an association study with a sample of 689 cocaine addicts to verify the influence of c.1021C>T polymorphism on the susceptibility to cocaine addiction. Genotypic and allelic distribution did not provide any evidence for association with cocaine addiction. [39]

Cocaine is also known for its effect of blocking serotonin reuptake from the synaptic cleft through the binding to the serotonin transporter 5HTT, which increases the level of this neurotransmitter at the neuronal synapses. [40]

Since altered 5-HT transmission is thought to increase susceptibility to dependence it is reasonable to question if polymorphisms in the 5HTT gene may contribute to the individual's risk for addiction, disease progression and response to treatment. The most studied functional polymorphisms of the *5HTT* gene are the *5HTT*-LPR (serotonin-transporter-linked polymorphic region) at the promoter region, which contains 14 (short, S) or 16 (long, L) copies of a 22-23 bp repeat element, and the *5HTT*-VNTR in intron 2, with four variants containing 9-12 repeats of a 16-17 bp unit (9R-12R). [40]

A recent study *in vitro* demonstrated that *5HTT*-LPR and *5HTT*-VNTR modulate the *5HTT* transcription in response to cocaine by altering the binding of different transcription factors and inducing chromatin modifications. Gene reporter experiments showed that the *LPR*-VNTR haplotypes *S-12R* and *L-10R* increased by two or six fold, respectively, the basal transcription levels in the presence of cocaine *in vitro*. [40, 43, 44]

A case-control association study conducted in a Spanish sample (n=504) aimed to evaluate the correlation between haplotype combinations of the *5HTT S-12R* and *L-10R* polymorphisms and the expression of the serotonin transporter after cocaine exposure. This study showed no evidence of an overrepresentation of any of these allelic combinations and no differences were observed neither in the presence or absence of psychotic symptons or comorbid dependence to other drugs. [40]

5.3 Genetic markers of opioid addiction

The effects of opioids and opiates are mediated primarily through the endogenous opioid receptor system, which includes receptors μ-opioid (MOR), δ-opioid (DOR), and κ-opioid (KOR). These three receptors are mainly expressed in the central and peripheral nervous

systems. Growing evidence from different reports has shown that these three opioid receptors mediate the analgesic effect and addictive properties of opioid drugs. Stimulation of MOR by opiates of abuse or endogenous ligands (e.g., enkephalins, endomorphines and dynorphins) inhibits transmission through the inhibitory GABA neurotransmitter system, thereby resulting in a disinhibition of the mesolimbic mesocortical dopamine pathways. [25, 45, 46]

Heroin and prescription opioids, such as oxycodone or hydrocodone, act primarily as MOR agonists with relatively short duration of action. [47]

The main active metabolites of heroin also act primarily as MOR agonists. Heroin enters the brain quickly and in high concentration. Once in the brain, heroin is rapidly converted to the biologically active metabolites morphine and monoacetylmorphine. This conversion may also occur in liver, in the first passage metabolism. These compounds bind MOR and relieve GABAergic inhibition of dopamine neurons. [47]

Methadone is a full MOR agonist and a weak NMDA receptor antagonist. Methadone metabolism is mediated by cytochrome P450 enzymes CYP3A4, CYP2B6 and CYP2D6. [47]

Opioid receptors have been the main focus of addiction research due to the its involvement in drug reward pathways. The rewarding effects of drug use are mediated by MOR and DOR activation, whereas KOR activation is mainly associated with aversion. [45]

Genes coding for proteins known to be involved in the pathophysiology of specific drug dependences are good candidates for genetic association studies of these neurodegenerative disorders. Genes encoding opioid receptors (*OPRM1*, *OPRD1* and *OPRK1*, which encode the μ-, δ- and κ- receptor, respectively) are among the most obvious candidates for investigating opioid dependence, but also other forms of substance abuse and addiction. [46]

The μ-receptor has been considered the primary target for opioid addiction and it has the highest affinity for morphine and its stimulation leads to pain relief and euphoria. Because the μ-opioid receptor is the primary target of opiates, genetic variations affecting its function became an important area of study when assessing the effects of pharmacogenetics on opiate addiction. Although MOR is considered the primary target for the rewarding effects of addiction, there are many MOR interacting proteins (MORIPs) that may modulate MOR function, among other factors. One of these MORIPs is DOR, suggesting that genetic variation in *OPRD1*, the gene encoding for DOR, may affect susceptibility to drug addiction. [25, 45, 46]

The MORIPs include opioid ligands and heterotrimeric G-proteins, which control receptor activation and downstream signaling, respectively. Other MORIPs are known to prevent

activation of MOR signaling or regulate the desensitization of MOR by blocking access to specific protein binding domains. The Wntless (WLS) homolog (Drosophila) protein was identified as a MORIP type of molecule, which is useful for model studies. A case-control association analysis has been performed to determine if common variants in the human *WLS* gene were associated with opioid or cocaine addiction in African-American and European-American populations. Nominally significant associations were found between 3 SNPs and opioid addiction. In the African-American population rs#3748705 was associated with addiction and rs#983034and rs#1036066 showed positive association in the European-Americans. None of the results for these SNPs were significant after false discovery rate (FDR) correction. [45]

Many functional variants have been identified in the *OPRM1*, being the most common c.118A>G (rs#1799971) in the coding region, exon 1, which causes the replacement of an asparagine residue by aspartic acid. The Asp40 residue results in a three-fold increase in β-endorphin binding compared with the asparagine's containing protein. The c.118G allele is most common in Asian populations (40-50%) and has a moderate frequency in European populations (15-30%), with very low prevalence in African populations. It has been shown that the c.118G allele is positioned within a haplogroup in a population-specific manner and is in high linkage disequilibrium (LD) with several distant variants that may have regulatory effects. Carriers of the c.118G allele show an elevated sensitivity to pain and reduced analgesic response to opioids. Homozygotes for this allele tend to need higher doses of oral morphine in treatment for cancer pain. [25, 47, 48]

A number of studies have reported positive associations between *OPRM1* functional variant c.118A>G and dependence of opiates, cocaine or alcohol. However, a meta-analysis that included the study of 28 samples showed no evidence for an association of this polymorphism with substance addiction. Although there was significant evidence of an association of substance dependence in some studies, the contradictory nature of the results from different authors complicates the interpretation and clinical biomedical significance of results. An alternative explanation is that the c.118A>G polymorphism is in linkage disequilibrium with another genetic alteration that conveys the primary effect on susceptibility to addiction. However haplotype analyses have also failed to yeld consistent findings. [45, 48]

The second most prevalent variant of the *OPRM1* is the c.17C>T SNP in the coding region, and it is found at frequencies that range from 0.5%–21% across different populations, but it

has been found mostly in populations with African ancestry. This SNP has been associated with a higher risk for opiate dependency. [25, 47]

The κ-opioid receptor gene (*OPRK1*) has also been implicated in response to opiates, and multiple variants in the gene have been reported. Preliminary evidences suggest that the SNP c.36G>T (allele T with frequency of 1–3% in Europeans) may be associated with an increased risk for opiate addiction. [25]

Although the primary actions of the δ-opioid receptor (coded y OPRD1 gene) manifest in nociception, some of its function lies in modulating the effects of μ-opioid receptor-directed opiates. Two coding variants of *OPRD1* have been studied for association with drug addiction: c.80G>T (rs#1042114) in exon 1 and c.921C>T (rs#2234918) in exon 3. The SNP c.80G>T was significantly associated with a higher likelihood of opioid dependency, and the haplotype that contains both SNPs c.80G>T and the c.921C>T had a significant risk effect on opioid dependency. [25, 45]

Mayer et al. (1997) reported a positive association between *OPRD1* c.921T>C and heroin addiction in a German population. These authors have found that both the C-allele and the C/C homozygotes were significantly more frequent in a sample of 103 German Caucasian heroin addicts, compared to control subjects. However, Franke et al. (1999) used both case-control and family-based designs in another German population, but found no evidence for association or linkage disequilbrium of the same variant with heroin or alcohol dependence. [46, 49, 50]

Zhang et al. (2008) analysed a sample of 1,063 European American subjects and reported that *OPRD1* variant c.80G>T in exon 1 may be associated with opiate dependence, since c.80G-allele was significantly more frequent in opiate dependent cases (21%) than in controls (13.2%). The high frequency of this allele in opiate dependent cases demonstrates that it could be a genetic risk factor for the disorder. Moreover, marker *OPRD1*1, which is located in the 5´-region and is 2,289 bp distant to c.80G>T, also showed a positive association with opiate dependence. Furthermore, the same authors found no evidence for association between *OPRD1* c.921T>C variant and drug dependence by individual marker analyses and thereby were not able to replicate Mayer's findings. [46]

Clarke et al. (2013) designed a study aiming to examine the contribution of rare coding variants of *OPRM1* to the risk for addiction because the majority of the association studies analyzing *OPRM1* and drug addiction have focused on common variants. These variants have an allele frequency above 5% in general population and, when associated with disease, typically confer a small to moderate amount of risk. Conversely, the rare variant hypothesis

states that a significant proportion of disease risk is due to low frequency variants that confer a much higher risk for disease. Rare variants of *OPRM1* gene were identified from the National Lung Heart and Blood Institute – Exome Sequencing Project and 4 SNPs were selected: rs#62638690 and rs#17174794 in European-American subjects and rs#1799971 and rs#17174801 in African–American subjects. The results showed that of the 2 SNPs genotyped in European-American cases, only rs#62638690 was significantly associated with drug addiction. The minor allele frequency in cases was 0.38% compared to 0.79% in the control population (p=0.02; OR: 0.47 CI 0.24-0.92), suggesting that it may confer a protective effect against drug addiction. In African–American cases neither rs#1799971 nor rs#17174801 were associated with drug addiction. The total rare variant burden in cases both in African–American and African–American was not found to be statistically different. [51]

Epidemiological studies indicate that non-genetic features contribute 40-60% to the risk of developing drug addiction. A relevant part of these are environmental and chemical/drug-induced factors, but other issues, such as epigenetic modifications (DNA methylation and chromatin remodeling) may also play an important role. The transmission of information not directly encoded in the DNA sequence is termed epigenetic inheritance. DNA methylation of cytosine residues in genomic DNA is a common epigenetic mechanism controlling gene expression and occurs through the insertion of a methyl group to cytosine residues in cytosine:guanine (CpG) dinucleotides by DNA methylation enzymes. CpG dinucleotides are often clustered in "CpG islands". [52]

Nielsen et al. (2009) reported a study on methylation of CpG sites in *OPRM1* gene promoter region in former heroin addicts stabilized with methadone treatment. It was found that in two of 16 CpG sites in a region of the *OPRIM1* gene promoter had significantly higher methylation in former heroin addicts than in controls. The two -18 and +204 CpG sites that were hypermethylated in the former heroin addicts are located in binding sites for the potential Sp1 transcription factor. It is possible that the hypermethylation at these sites reduces expression of the *OPRM1* gene in former heroin addicts. Future studies may determine whether the hypermethylation was due to methadone maintenance pharmacotherapy, heroin or if such methylation state might have been inherited through genomic imprinting. [52, 53]

A genome-wide association study approach, which included genotyping 10,000 variants simultaneously in 104 former severe heroin addicts and 101 controls, was used to identify genetic variants in genes involved in the vulnerability to develop heroin addiction. The

authors reported that when allele frequency was analyzed for association with heroin addiction, the strongest association was with the autosomal variant rs#965972, located in a Unigene cluster of unknown function and in a region predicted to have high regulatory potential and with rs#1986513, which is located in a region of high conservation in mammals. The three variants exhibiting the strongest association with heroin addiction by genotype frequency were rs#1714984, located in an intron of the gene for the transcription factor myocardin, rs#965972 and rs#1867898. One haplotype genotype pattern (AG-TT-GG) was found to be positively associated with developing heroin addiction (odds ratio= 6.25) and explained 27% of the population attributable risk for heroin addiction in this cohort. [52, 53]

In a hypothesis-driven multi-gene study, 1,350 variants were screened in 130 candidate genes in subjects with European ancestry. The case subjects were former severe heroin addicts undergoing methadone maintenance treatment. This approach is based on physiological hypotheses and the genes were selected based on their function and related pathways. Nine variants, in six genes, showed nominal significant associations but none of those remained significant after adjustment for multiple testing. These variants were in non-coding regions of the genes *OPRM1*, *OPRK1*, *OPRD1* and , *5HTR3B*, among others. [52]

Gelernter et al. (2013) reported results for a GWAS for opioid dependence in two different population groups in the United States. They have also used data from the Study of Addiction: Genetics and Environment (SAGE). Several of the top-ranked genes encode proteins that participate in potassium and calcium signaling pathways. Although signaling related genes have been studied in addiction biology, they were not previously considered key candidates for molecular genetic studies. The loci *KCNC1* and *KCNG2*, containing some of the most significantly associated SNPs, encode potassium voltage-gated channel subunits. Potassium-calcium signaling is involved in coupling neuronal signals to vasodilation in the brain and opioids can regulate calcium conductance via increasing potassium conductance in MOR receptors. One of the genes in the calcium signaling pathway that is strongly associated with opiate dependence risk, *CAMK2B*, codes for a protein that modulates activation of ionotropic glutamate receptors. These pathways may interact to produce biologically important effects, both on behavior after an initial exposure to opioids and on impaired control over use after chronic exposure. [54]

5.4 Genetic Markers of Nicotine Addiction

The development of nicotine dependence is the last step in a sequence of behavioral events that starts with the initiation of cigarette use. There are at least three steps in this process: the transition from never smoking to the initiation of cigarette use; the conversion from experimental smoking to the establishment of regular smoking behavior, and finally the development of nicotine dependence among smokers. Each step in this pathway of smoking represents a potential point for intervention to prevent the onset of nicotine dependence, and different genetic and environmental factors influence the progression through each stage. [55]

Smoking begins with the first cigarette and the next step in the development of dependence is the transition from an experimental smoker to a "smoker", which is defined as an individual who has smoked 100 or more cigarettes, a threshold used in many large-scale epidemiological studies. [55]

The progression from smoking to nicotine dependence is influenced by genetic and environmental factors. A cluster of symptoms define nicotine dependence, which includes tolerance to nicotine (the use of larger amount of substance to obtain the same effect, which is consistent with smoking 20 or more cigarettes a day), withdrawal symptons, and use of cigarettes despite social restrictions and health consequences. However, not all smokers develop nicotine dependence. About half of current smokers are dependent on cigarettes and many others have some symptoms of dependence. There is a third group of smokers who have no addiction symptoms, a group called "chippers". In contrast to the nicotine dependent smokers who smoke daily and generally are heavy smokers, frequently smoking 20 cigarettes a day, chippers smoke a few cigarettes a day and may not smoke daily. In genetic studies, chippers represent a unique contrast sample to smokers who develop nicotine dependence. [55]

Nicotine is the main reinforcing component of tobacco smoke and is highly addictive. It binds and activates the nicotinic acetylcholine receptor in the VTA, which facilitates dopamine release in the shell of the NAc, thereby activating the mesolimbic brain reward pathway. These receptors are pentameric molecular assemblies of nicotinic acetylcholine receptor (nAChR) subunits, which are coded by a family of distinct cholinergic nicotinic receptor (CHRN) genes. There is a substantial genetic contribution to various aspects of smoking, including initiation, progression, maintenance, amount smoked, and the ability to quit. Twin

studies estimated that the heritability of nicotine dependence is approximately 50%. [25, 55, 56]

Similar to other diseases with complex etiologies, each gene probably contributes only part of the genetic susceptibility to nicotine dependency, and interactions between multiple genes ultimately contribute to the risk for smoking. [25]

Several large-scale studies have identified the region on chromosome 15 that includes genes coding for the family of α5α3β4 nicotinic receptors, as being associated with the risk for a smoker becoming a nicotine dependent. [55]

In order to identify alleles associated with nicotine dependence, as number of cigarettes per day (CPD) regularly smoked, genetic analysis was conducted in two large European populations (~7,500 subjects) using wide genome analysis (WGA) techniques. Berrettini et al. (2008) identified independently the same genetic association on chromosome 15, pointed by Bierut et al (2009), associating *CHRNA3* and *CHRNA5* with the risk to nicotine dependence. Due to linkage disequilibrium in the *CHRNA3-CHRNA5* region, it is possible that the causative alleles may lie within either or both of these genes, which code for nicotinic receptor subunits. [55, 57]

Saccone et al. (2009) analyzed 226 SNPs covering the complete family of 16 The *CHRN* genes, which encode the nicotinic acetylcholine receptor (nAChR) subunits were analysed in a sample of 1,050 nicotine-dependent cases. A significant association was found in three gene clusters: two distinct *loci* in the *CHRNA5-CHRNA3-CHRNB4*, one *locus* in the *CHRNB3-CHRNA6* and a novel *locus* in the *CHRND-CHRNG*. The two distinct *loci* in *CHNA5-CHRNA3-CHRNB4* are represented by the non-synonymous SNP rs#16969968 (c.1192G>A) in *CHRNA5* and by rs#578776 (c*546C>T) in *CHRNA3*. [56]

Besides the dopaminergic system, nicotinic acetylcholine receptors have been implicated in nicotine reward and dependency. Two non-synonymous SNPs on exon 5 (rs#1044396/c.1629G>T and rs#1044397/c.1659G>A) of the *CHRNA4* gene, encoding for the α4 subunit of the nicotinic acetylcholine receptor, were associated with a lower risk of nicotine dependence in Chinese men, which was recently replicated in a European population. A G>A variant, rs#2236196, in the 3'-untranslated region of *CHRNA4*, was associated with increased subjective effects of nicotine and higher risk of nicotine addiction. [25]

Several other genetic variations, in genes coding for nicotinic acetylcholine receptor subunits, may also be associated with nicotine dependency. Specifically, variation in the *CHRNA5-CHRNA3-CHRNB4* gene cluster on the long arm of chromosome 15 was

associated with a higher risk for lung cancer, smoking a higher number of cigarettes, and nicotine dependency. Although some studies suggest that this *locus* is not associated with smoking behaviors, recent evidence proposes that multiple SNPs in this gene cluster are associated with a higher number of cigarettes per day and greater nicotine dependency. Specifically, the non-synonymous SNP rs#16969968 in *CHRNA5* appears to be the most promising variant for biological contribution to nicotine dependence, since it has been associated repeatedly with higher nicotine addiction, and individuals with the variant allele (c.1192G>A) in this SNP had a higher smoking intensity. This SNP causes a change in amino acid 398 from asparagines (encoded by the G allele) to aspartic acid (encoded by A, the risk allele) which results in the change of an amino acid in the α5-nicotinic receptor protein and the insert of this of this single amino acid in *in vitro* models showed a change in receptor function. [25, 55, 58]

Both rs#16969968 and rs#578776 contribute to the risk of developing nicotine dependence equally in men and women. The A-allele of rs#16969968 (c.1192G>A) increases the risk of becoming nicotine dependent, compared to non-dependent by 30%; while the A-allele of rs#578776 (c.*546C>T) decreases the risk of developing nicotine dependence by more than 30%. However, different implications of the risk accountable from these genetic variants contribute may occur, because of the variation in allele frequencies among ethnic populations. [55]

The findings regarding the chromosome 15 region have revealed different allele frequencies according to ethnicity. [55]

The SNP rs#16969968 is common in the European population (minor allele frequency – MAF= 0.42), but it is rare in Asians (MAF=0.01-0.03) and it is absent in Sub Saharan African populations (MAF=0). [55]

The second association, related to rs#578776, has also different allele frequencies in different populations. The highest frequencies were identified in Asians (MAF=0.80) and in Sub-Saharan population (MAF=0.65), while the lowest was in the Europeans (MAF=0.24). Considering that the T allele has been associated to a protective effect against nicotine dependence, it may have a superior influence on the African-American and Asian populations. [55]

Genes that alter the pharmacokinetics of nicotine metabolism are also involved in smoking phenotypes. In the first phase metabolism, 80% of nicotine is metabolized to the bioactive metabolite cotinine, mainly by cytochrome P450 2A6 (CYP2A6) and variability in rate of metabolism contributes to vulnerability to tobacco dependence and response to smoking

cessation treatment. Cotinine is then metabolized to 3'-hydroxycotinine (3HC) exclusively by CYP2A6, so the ratio of conversion of cotinine to 3HC may be used as a phenotypic marker for CYP2A6 activity. [25, 59, 60]

Nicotine and cotinine are also metabolized by glucoronidation via UGT 1A4, 1A9 and 2B10. Although glucoronidation is usually a minor pathway of nicotine metabolism, in subjects with low CYP2A6 activity, it may be a major determinant of nicotine clearance. [59]

The CYP2A6 is genetically polymorphic, with over 37 alleles identified for this gene, and this variation influences the metabolism of nicotine. Genetic slow metabolizers have longer nicotine half-live, resulting in prolonged nicotine plasma levels. They also have reduced withdrawal symptoms and higher quitting rates. Genetic variations in CYP2B6, another member of the cytochrome P450 family, can also alter smoking behaviors. Individuals with one or more copies of *CYP2B6*5* allele (c.1459C>T) were shown to have greater craving and a higher relapse rate. [25, 59]

6. Pharmacogenetics and Drug Addiction Treatment

6.1 Alcohol addiction treatments and Pharmacogenetics

There are currently three FDA-approved medications for the treatment of alcohol dependence: naltrexone (both oral and depot), acamprosate and disulfiram. A fourth drug, topiramate, has shown compelling evidence of efficacy in two randomized controlled trials and frequently is used "off-label" for alcohol dependence. [32]

In Portugal, there are four approved medications for alcohol dependence, namely acamprosate, disulfiram, naltrexone and the newest nalmefene (Selincro®). [61]

Disulfiram, rarely prescribed since it has potentially serious side effects, has been used as a drinking deterrent because, by blocking acetaldehyde metabolism in the alcohol metabolic pathway, it produces the very unpleasant flushing syndrome. Naltrexone, a μ-opioid receptor antagonist, has been shown to have a modest effect on drinking outcomes. Acamprosate, a weak NMDA antagonist, helps maintaining abstinence to alcohol through a mechanism that may involve an interaction with glutamate and GABA neurotransmitter central systems. Nalmefene is an opioid system modulator, with antagonist activity at the μ and δ receptors and partial agonist activity at the k receptor. [62, 63]

Many patients do not respond to these medications, and side effects often limit usefulness. Efforts to try to predict treatment response and side-effect risk of specific medications for alcohol dependence treatment with pharmacogenetic analyses have begun. The opioid receptor antagonist naltrexone is the medication most studied in pharmacogenetic analyses of alcohol treatment trial data. [64]

OPRM1 is a key candidate gene, since β- endorphin and the μ-opioid receptor have been shown to play an important role in the rewarding or reinforcing effects of alcohol. A polymorphism in the μ-opioid receptor gene OPRM1, c.118A>G (p.Asn40Asp; rs#1799971) produces a threefold increase in β-endorphin binding affinity and potency. Therefore, this is the best studied genetic variant with high relevance to alcoholism treatment. [29]

Evidence from clinical trials suggests that the presence of the variant 108G allele of rs#1799971 may predict better treatment response to opioid receptor antagonists, such as naltrexone. [29, 64]

Gelernter et al. (2014) looked for pharmacogenetic effects of several opioid-receptor gene polymorphisms, including rs#1799971, in an analysis of a large subgroup of subjects from the

VA Cooperative Study of naltrexone (vs placebo) for the treatment of alcohol dependence. In this subset, naltrexone was significantly better than placebo for preventing relapse to any heavy drinking, but no effect of opioid-receptor polymorphism moderated the efficacy of naltrexone on relapse rates [64]

In general, alcoholics show reduced central dopaminergic sensitivity that is also associated with poor treatment outcome. However, alcoholics carrying the 108G allele have shown significantly higher central dopaminergic receptor sensitivity, after one week of abstinence that was not seen before detoxification. These findings are in line with another study showing that naltrexone-treated alcoholics carrying 108G allele, had significantly lower relapse rates and took longer to go back to heavy drinking, compared to AA homozygotes. Likewise, a meta-analysis using a random effects model of six studies, published between 2002 and 2009, that investigated the association between c.118A>G and response to naltrexone in alcoholics determined that carriers of G allele had modestly lower relapse rates that AA homozygotes (OR = 2.02, 95% CI = 1.26–3.22), but there was no difference in abstinence rates . Three placebo-controlled trials have found that alcohol dependent individuals with a 108G allele have better clinical responses to naltrexone, including lower relapse rates than those with the A allele. [29, 65]

In a 12 week, randomized clinical trial of naltrexone to reduce drinking in 158 problem drinkers, G allele carriers were at increased risk of drinking more when the desire to drink was relatively high, and this was attenuated by naltrexone. However, another study did not find an effect of this polymorphism on drinking reduction in non-treatment seeking alcoholics. [29]

One relapse-prevention study tested the hypothesis that naltrexone response would be associated with allele variations in genes coding for dopaminergic and opioidergic proteins, and that acamprosate response would be associated with glutamatergic and GABAergic genetic variations influencing function. Treatment-seeking and treatment-non-seeking Dutch alcoholics (n=126) were randomly assigned to either naltrexone 50 mg daily or acamprosate 1.3–2.0 g daily and were exposed to alcohol craving cues. The efficacy of acamprosate on craving reduction was enhanced, depending on the C-allele frequency of the SNP of *GABRA6* gene, whereas the efficacy of naltrexone was enhanced depending on the frequency of the T allele. A1- allele homozygotes of the TaqIA *DRD2* polymorphism did better with acamprosate; those with the A2 allele did better with naltrexone. Both results suggest an important role of genetics influencing reward circuitry for treatment of alcohol dependence. [64]

Additional investigations focused on the *ANKK1* Taq1A (rs#1800497) polymorphism, as well as on genes involved in GABAergic glutamatergic and opioidergic neurotransmission. In a randomized, double-blind placebo controlled study of 108 Dutch individuals, an A2A2 genotype at the Taq 1A polymorphism of *ANNKK1* gene, was associated with increased naloxone efficacy, while an A1A1 genotype was associated with an increased ability of acamprosate to blunt cue induced cravings. [65]

6.2 Cocaine addiction treatments and Pharmacogenetics

Cocaine dependence is common and has social and economic impact but it has no Food and Drug Administration (USA) approved specific pharmacotherapy. [66]

Although a number of innovative pharmacological approaches, such as antidepressants, dopamine agonists and anti-epileptic drugs, have had limited success in reducing cocaine use, disulfiram has shown some initial promise in treating cocaine dependence. [66, 67]

Since cocaine addiction has a strong genetic basis, pharmacotherapy for this relapsing brain disease should be based on a molecular genetics approach. [66]

Applying a molecular genetics approach to disulfiram might involve its inhibitory action on the copper-containing glycoprotein enzyme DBH. [66]

According to twin and family studies, plasma levels of DBH vary between unrelated individuals and some of these differences are due to polymorphisms close to the *DBH* gene. A few studies link the -1021C>T variant to differences in circulating DBH levels. Several studies indicate that this variant has functional impact, by altering transcription and leading to decreased plasma levels of DBH. Individuals that are homozygous for the T allele have the lowest levels of plasma DBH activity. [41, 66]

Having in mind the different DBH activities depending on the genetic variant and the possibility that disulfiram might not be an effective treatment for cocaine dependence, depending on *DBH* polymorphisms, a clinical trial was conducted to explore this potential matching. [66]

A study that included 74 cocaine dependent subjects were randomly treated with disulfiram 250 mg daily or placebo, while stabilized on methadone maintenance at 60mg daily. During the 10 weeks of the study, urine samples were obtained and tested for the presence of cocaine metabolites. The results showed that patients having two of the alleles associated to normal activity of DBH (CC, rs#1611115) had a good response to disulfiram, with cocaine

positive urines decreasing from 84% to 56%. On the other hand, those with genotypes encoding for lower activity (CT and TT) showed no difference from the placebo. This study provides evidences that genotyping *DBH* could be used to identify a group of individuals for which disulfiram treatment might be an effective treatment for cocaine dependence. [66]

Recent evidence suggests that stimulation of the noradrenergic system contributes to reward and reinforcement from the drug in individuals abusing of cocaine. Dopamine transporter knockout mice continue to self-administer cocaine, suggesting that blockage of DAT alone is not sufficient to account for the reinforcing effects of cocaine suggesting that other neurotransmitter systems must contribute and be involved in the process. Moreover, norepinephrine transporter knockout mice display a reduced response to acute cocaine administration. A functional coupling of the noradrenergic system to the dopaminergic system may be mediated through the activation of α1A-adrenoceptors, contributing to cocaine-induced increase in synaptic levels of norepinephrine and subsequent increase in firing of dopamine neurons in VTA and PFC. Preclinical evidence data allowed demonstration that pharmacologic blockade of noradrenergic system attenuates reinstatement of cocaine-seeking behavior in rats. [34]

Disulfiram inhibits DBH, leading to decrease of norepinephrine levels, which leads to a reduction in stimulation of α1A-adrenoceptors. Based on this assumption and in order to identify clinical subpopulations, in which the efficacy of disulfiram may be improved, D. Shorter et al. (2013) examined cocaine dependent patients based upon *ADRA1A* genotype. The *ADRA1A* gene codes for the α1A-adrenoceptors and has a polymorphism, rs#1048101, in exon 2, coding for the substitution of an arginine (ARG) for a cytosine (CYS) at codon 347 of the C-terminus, which may alter the functional activity of this receptor. The aim of this study was to evaluate whether cocaine addicted patients, carriers of the T allele (TT/ TC), Cys347, would have a different response to disulfiram, compared to patients homozygous CC, Arg347. [34]

The results showed that cocaine consumption decreased from 80% to 59% in disulfiram group. Furthermore, when the sample is separated into two genotype groups (CC versus TT/ TC), cocaine positive urine rates were different between the treatment groups for individuals carrying the T allele, but did not differ for those with CC genotype. This result suggest that disulfiram reduced the percentage of cocaine positive urines among individuals with the CYS conformation of the ADRA1 receptor, but not in those with the ARG substitution, related to SNP rs#1048101. [34]

These results, combined with the prior study showing that disulfiram reduced cocaine consumption in carriers of *ADRA1* allele CYS and with *DBH* -1021C>T polymorphism CC (wild type). [34, 66]

6.3 Opioid addiction treatments and Pharmacogenetics

Methadone administration and maintenance is the gold standard therapy for heroin addiction and successful treatment relies to a certain extent on individual dose optimization. Methadone is a synthetic opioid that is administered as a racemic mixture of (R)- and (S)-enantiomers, although it is the (R)-methadone that accounts for the opioid receptor neurochemical activation. Methadone is rapidly absorbed with peak plasma concentrations 2-4h after oral administration and is metabolized primarily in the liver. As previously referred, the major methadone-metabolyzing enzymes are cytochrome P450 CYP3A4, CYP2D6 and CYP2B6. The large inter-individual variation in the pharmacokinetics and response to methadone may be explained in part by the functional impact of some of these genetic variants. [52]

Concerning *CYP2D6* genotypes, the general population is comprised of extensive, intermediate, poor and ultra-rapid metabolizers. Ultra-rapid metabolizers were found to have unsuccessful methadone treatment therapy, but it has been reported that they have good response to buprenorphine that is not significantly metabolized by CYP2D6 enzyme**.** [52]

Methadone is a substrate of P-glycoprotein 170 (P-gp). The P-gp is a member of the subfamily B of the ATP-binding cassette (ABC) superfamily. It has a significant role in drug pharmacokinetics. It is encoded by the high polymorphic *ABCB1* gene with variation in allele frequencies among different populations. Genetic variability in the *ABCB1* gene may influence methadone distribution by altering P-gp expression and function. The most studied SNP is the synonymous sequence variation c.3435C>T (rs#1045642) that has showed to be related to lower *in vivo* duodenal P-gp expression and also lower mRNA expression in human liver samples. Variants c.1236T (rs#1128503, c.1236T>C), c.2677T (rs#2032582, c.2677T>A) and c.3435T (rs#1045642, c.3435C>T) were reported to minimize P-gp activity *in vitro* in a substrate-specific manner. [52]

A study of 179 European individuals shows that carriers of the *CYP2B6*6* allele (rs#3745274) had an increased risk of prolonged QTc interval when treated with

methadone. A second study of 245 individuals undergoing methadone therapy has shown that the *CYP2B6*6* allele (rs3745274) was associated with higher levels of the drug. However there was no effect on the success of treatment. [65]

Buprenorphine is a synthetic opioid with primarily MOR partial agonism and modest κ-opioid receptor activity. It is available for heroin detoxification as a single agent but marketed primarily in the United States in a combination preparation with naloxone for maintenance therapy. Buprenorphine has a much shorter terminal half-life (3 to 5h) than methadone (24 o 36h), but dissociates slowly from MOR over 24 to 48h, allowing for daily or even once every 3-day dosing. Because it is a partial agonist with strong MOR affinity, buprenorphine can induce withdrawal symptoms in moderately to highly opiate tolerant individuals. [17]

The standard medications used in the treatment of opiate dependence, such as methadone and buprenorphine, are all primarily metabolized by CYP3A4. Polymorphisms that affect CYP3A4 function may influence the efficacy of these treatment agents. [17]

On the other hand, treating mixed drug abusing consumptions, which are very common, is a challenge in addiction therapeutics. [46]

The three opioid receptors also play important roles in the reinforcing properties of non-opioid drugs, such as cocaine, and alcohol. Cocaine is thought to act primarily at the dopamine transporter and the dopamine system is intimately interconnected with the endogenous opioid systems. Repeated administration of cocaine evokes a down-regulation of δ-receptor density in the NAc. It was found that a single cocaine exposure enhanced both μ-receptor and κ-receptor aversion through a circuit involving the ventral tegmental area, in which dopamine neurons are abundant. Furthermore μ- and δ-receptor agonists sensitize animals to the rewarding effect of cocaine and alcohol, whereas κ-receptor agonists inhibit this function. Moreover, pharmacological blockage of the endogenous opioid system by μ- and δ-receptor antagonists prevent ethanol from activating the dopamine system and reduces ethanol craving and consumption. Thus, μ-and δ-receptor antagonists can be useful in the treatment of alcohol dependence. Naltrexone is thought to exert its action primarily by blocking the μ-receptor and is one of the rare approved pharmacotherapies for alcohol and drug addiction. [46]

6.4 Nicotine dependence treatments and Pharmacogenetics

Currently, three classes of medications have been approved for smoking cessation: nicotine replacement products (patch, gum, spray and inhaler), bupropion and varenicline. [59]

The main action of nicotine replacement medication aims to replace partially the nicotine from cigarettes to produce the relief of withdrawal symptoms when a person stops tobacco consumption and to reduce craving. Amelioration of these symptoms is observed with relatively low blood levels of nicotine. [59, 68]

The mechanism of action of bupropion as a support in smoking cessation is unknown. It is presumed that its effect is mediated by a noradrenergic and/or dopaminergic mechanism, since bupropion increases brain levels of dopamine and norepinephrine, simulating the effects of nicotine on these neurotransmitters. Bupropion also has some nicotine receptor-blocking activity. [59, 69]

Varenicline was synthesized with the goal of developing a specific antagonist for the $\alpha4\beta2$ nAchR. Varenicline is a partial agonist of $\alpha4\beta2$ receptor in vivo and produces less of a response than nicotine (~50%) but, at the same time, it blocks the effects of any nicotine added to the system. Clinical trials have showed that varenicline is superior to bupropion when evaluating smoking abstinence rates. [59, 69]

A number of pharmacogenetic studies have been carried, focusing primarily on candidate genes related to nicotine reward and nicotine metabolism pathways. [59]

The gene *DRD2* has been investigated and it was found that women with the *DRD2-ANKK1 Taq1 A1* variant presented a considerable benefit from nicotine patches. In a double-blind randomized clinical trial with 368 current smokers, two other *DRD2* polymorphisms (c.957C>T - rs#6277 and -141C Ins/ Del rs#1799732) have been demonstrated to influence treatment response to nicotine replacement therapy. Smokers carrying the allele -141Cdel had higher smoking quit rates on nicotine replacement therapies compared to homozygous for the -141Cins allele. The same study also demonstrated that for the c.957C>T variation, smokers with the C-allele were less likely to be abstinent at the end of treatment than smokers with TT genotype. [68, 70]

Novel CYP2A6 genetic variants have been discovered, but only some of these variants demonstrated to modify enzyme activity. While the *CYP2A6*2* allele and *CYP2A6*4* are fully inactive, others including *CYP2A6*9* and *CYP2A6*12* result in decreased enzyme activity. Slow metabolizers are those individuals carrying at least one copy of the inactive variants (*CYP2A6*2* and *CYP2A6*4*) or having two *CYP2A6*9A* or *CYP2A6*12A* alleles. [68]

Smokers that are slow metabolizers with the *CYP2A6*4* allele showed higher relapse rates following nicotine patch, compared with smokers with *CYP2A6*1*. [68]

The *DRD2* polymorphism -141CIns/Del has also been analyzed for bupropion treatment. Smokers who were homozygous for the -141CIns allele showed higher abstinence rates with bupropion than smokers with the -141CDel allele. Comparing the data regarding this polymorphism with nicotine replacement therapy, these results suggested that smokers with InsC/InsC genotype may respond better to bupropion, while individuals that are homozygous DelC/ DelC may have a better response to nicotine replacement therapy. [68, 70]

O'Gara and colleagues (2007) conducted a study of the influence of SLC6A3polymorphisms, related to dopamine transporter DAT1, to the outcome of smoking cessation treatment with bupropion or nicotine replacement therapy. Smokers carrying the 9-repeat allele at 3' UTR VNTR, or the 2-repeat allele at VNTR in intron 8 were more likely to quit smoking after treatment. However, these results were only observable one week after smoking cessation. [68, 71]

Bupropion is metabolized to a primary metabolite, hydroxybupropion, by CYP2B6. Some polymorphisms of *CYP2B6* gene have been shown to have no influence on bupropion pharmacokinetics. For example, smokers homozygous CC for *CYP2B6*5* (c.1459C>T) were more likely to be abstinent from smoking with bupropion treatment, than those carrying the T-allele (slow metabolizers). In the same study, T-allele was reported to be associated with higher relapse rates and greater increases in cravings [68]

The *CHRNA5-CNHRNA3-CHRNB4* cluster variants have been less consistently associated with cessation outcomes than with smoking heaviness measures. However, some studies have shown association between the *CHRNA5-CNHRNA3-CHRNB4* region and successful in smoking cessation. It was also found that the same genetic risk variants that contribute to smoking heaviness and nicotine dependence also predicted smoking cessation [72].

The Pharmacogenetics of Nicotine Addiction Treatment (PNAT) Consortium was formed in 2005, aiming to identify the role of pharmacokinetic and pharmacodynamic gene variation on nicotine dependence and metabolism phenotypes, with focus on smoking cessation and medication response. In this context, Bergen et al. (2013) conducted an association analysis of the nAChR candidate gene variations with abstinence at end of treatment (EOT) and 6 month after the quit day in 2,633 treatment-seeking smokers. The results showed that "risk" haplotype (rs#588765 - c.106+7258T>C and rs#1051730 - c.645C>T) previously associated with smoking heaviness was significantly associated with increased abstinence in the nicotine

replacement therapy group, but not with other therapies, such as bupropion or varenicline. [73]

7. Conclusion and Future Perspectives

Drug addiction is a complex disease which is influenced by environmental and genetic factors. It is a neurodegenerative disorder that affects millions of people worldwide and causes social impairment and has important associated co-morbidities. Drug addiction remains an unsolved health issue and has limited treatment options currently available. Furthermore, the existing medications were not developed having a thorough knowledge of genetic and neurobiological causes of the disease. Accordingly, these are a few reasons why a huge effort has been made to evaluate the genetic causes underlying this disease and to go further in the understanding of the susceptibility of certain individuals to substance abuse and heterogeneity in therapeutic response.

A significant attempt to reach a deep knowledge has been made in the past 20 years when the twin and adoption studies were carried out to evaluate if heritability could play a role in dependences. Since then, science and technique has evolved, allowing a faster development in this area. Starting with PET scans that allow having a better and enhanced understanding of brain areas involved in reward circuits; also, more refined and effective DNA sequencing techniques, which enable the identification of genetic variations involved in drug addiction, have been contributing for the significant amount of data produced leading to a more detailed understanding of the neurobiologic and genetic etiology of drug addiction.

Many GWAS for several drug addictions were conducted. A few SNPs achieved statistically significance, being considered genome wide-significant and numerous candidate genes have been implicated in the etiology and response to treatment. However, in several studies the results were unexpectedly conflicting or did not reach statistic significance, which brings some confusion to the field and compromises the effective set up of rational clinical tools based on genetic and neurobiological data.

We have reached the point where the undertaking has given some results and the necessary techniques are available.

So, what is next?

More replication data is needed concerning some genetic variants to allow the identification of functional variants, but also the need for larger population samples has become clear for detecting small effect variants from the many genes accountable for addiction.

It is also important to take a better look at study design, such as the selection of samples, that must be carried carefully to prevent population stratification and also a consistent phenotype should be chosen.

In some studies, the results seem to fail because of the control group (never users *vs* former users); thus, this group should be thoroughly selected to avoid bias in the results.

With the upcoming of NGS genetic analysis, new challenges will rise, such as data storage and the need of tools for quality control of such amount of data, leading to the need of bioinformatics expertise in the working teams.

Genetic information has proved to be important not only in what concerns the cause of the disease, but also in the response to treatment, with clinical trials showing that genetic variants can influence the clinical response to a high extent. More clinical trials concerning drug dependence treatment should be conducted to improve the efficacy of clinical response, and taking into account genetics and functional phenotypic variations.

The main goal of pharmacogenomics of addiction, which is the development of medication based on the deep knowledge of the genetic underlying the causes of the disease – the truly personalized medicine, seems yet to be far, since this development should begin with validated functional variants.

However, efforts are being made in that direction, contributing for improving understanding and expecting to improve quality of life of patients.

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